THE UNIVERSITY
of EDINBURGH

Identification of novel Focal Adhesion Kinase binding partners and their biological functions in cancer cells

Ketevan Paliashvili

Doctor of Philosophy – The University of Edinburgh – 2015

Table of Contents

Figures

Tables

Acknowledgements

Firstly, I would like to thank my supervisor Prof Margaret Frame for the guidance, encouragements and advise she has given me throughout my times as her student. I would also like to thank all members of the Frame lab. In particular, a big thanks to Dr Emma Sandilands, who has always being there for me, for her assistance with experiments. I would like to say a special thank you to Dr Bryan Serrels for his continued support from day one, keeping me going when times were tough and offering invaluable advice. Thanks to Dr Hitesh Patel for offering friendship as well as scientific advice.

I would like to thank my family for their continued support and encouragement. Thanks to my mum for always being there when I needed, and big thanks to my daughter, Nicola for understanding while I spend many ours in the laboratory.

Finally, I would like to thank the Medical Research Council for providing the funding that allowed me to undertake this research, and also for giving me the opportunity to attend the conferences.

Declaration

I hereby declare that the work in this thesis was performed personally unless stated otherwise and it has not been submitted for any other degree or professional qualification.

Abstract

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that localises to focal adhesions. FAK is crucial for many cellular processes that are disturbed in malignancy, including proliferation, cell cycle, cell survival, adhesion, and migration. Mouse models have shown that FAK is involved in tumour formation and progression. Other studies demonstrated a functional correlation between FAK expression, tumour progression and malignancy in human cancer, making FAK a potentially important therapeutic target. Several FAK inhibitors have been developed most of which target the FAK kinase function. However, FAK may predominantly act as a scaffolding molecule rather than as a kinase, therefore, disruption of FAK's interaction with protein binding partners could be a good strategy to inhibit some cancer processes.

The identification and characterisation of novel FAK interactions may help to uncover important molecular mechanisms that, in turn, regulate key cellular processes involved in tumour formation and/or progression. Disruption of their function, or inhibition of their binding to FAK, will define their roles and identify whether they are good anti-cancer targets.

In this thesis work, I set out to identify novel binding partners of FAK, and study the role of a sub-set of these in tumour biology by impairing them in squamous cell carcinoma cancer cells *in vitro*. To do this I employed protein microarray and phage display methodologies using FAKΔ375 and FAK-FERM recombinant proteins as bait, respectively. I identified a number of novel proteins that interact directly with FAK. Then I set out to characterise some of these proteins.

The first of these, Axl, is a protein receptor tyrosine kinase that has previously been linked with tumour progression and metastasis in number of human cancers. I confirmed the interaction between FAK and Axl in SCC cells and showed that the FAK-Axl interaction is predominantly a scaffolding function of FAK, which seems to be unregulated, at least by any of the major phosphorylation events characterised for FAK. I also found that Axl controls cell spreading, cell polarisation and invasive migration in this cancer cell lines.

The second protein I characterise is the autophagy protein Ambra1. I found that Ambra1 is required for selective targeting of active Src to the autophagy pathway – a process that SCC cancer cells use when they are under adhesion stress, such as when FAK is deleted.

Thus, Axl and Ambra1 are potentially important proteins in SCC biology. They bind to FAK and function at cell adhesions to promote cancer-associated cellular processes. Analysis of FAK binding proteins may be a useful strategy to discover proteins that function in various aspects of cancer cell behaviour.

Abbreviations

µg	micrograms
µl	microlitre
µM	micromolar
nM	nanomolar
α	alpha
A	alanine
Ambra1	activating molecule in Beclin1 – regulated autophagy
Arp2/3	actin related protein 2/3
ASAP1	Arf-GAP containing a SH3 domain, ankyrin repeats, and PH domain
Atg	autophagy related
ATP	adenosine-5'-triphosphate
β	beta
Bif-1	Bax-interacting factor-1
bp	base pair
BSA	bovine serum albumin
CADTK	calcium-dependent tyrosine kinase
CAP	c-Cbl associated protein
cDNA	complementary DNA

CML	chronic myeloid leukemia
C-terminal	carboxy-terminal
D	aspartate
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbeccos Modified Eagles Medium
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinases
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FBS	foetal bovine serum
FERM	functional ezrin radixin myosin
FIP200	FAK interacting protein of 200 kDa
FGFR	fibroblast growth factor receptor
FRNK	FAK related non-kinase
ɣ	gamma
GAPDH	glyceraldehydes 3-phosphate dehydrogenase

GRAF	GTPase regulator associated with FAK
Grb7	growth factor receptor bound protein 7
HER2	human epidermal growth factor receptor 2
HUVEC	human umbilical vein endothelial cell
IF	immunofluorescence
IP	immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K14	keratin 14
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEF	mouse embryo fibroblast
MEM	Modified Eagles Medium
MMP	matrix metalloproteinase
NES	nuclear export signal
NLS	nuclear localisation signal
NRTK	non-receptor protein tyrosine kinases
NSCLC	non-small cell lung cancer
4-OHT	4-hydroxy-tamoxifen

P	proline
PBS	phosphate buffered saline
PBS1/2	paxillin binding site 1/2
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3 kinase
PLCɣ	phospholipase C gamma
PTB	phosphor-tyrosine binding
PTEN	phosphatase and ten-sin homolog
PTP	protein tyrosine phosphatase
Pyk2	protein tyrosine kinase 2
qRT-PCR	quantitative reverse transcriptase – polymerase chain reaction
RACK1	receptor for activated C kinase 1
RAFTK	related adhesion focal tyrosine kinase
RNA	ribonucleic acid
RT-PCR	quantitative reverse transcriptase – polymerase chain reaction
RTK	receptor tyrosine kinase
SCC	squamous cell carcinoma

SEM	standard error of the mean
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFK	Src family kinases
SH	Src homology
Shc	Src homologous and collagen-like protein
siRNA	short interfering RNA
SRB	sulforhodamine B
TAM	Tyro-3, Axl, and Mer
uPA	urokinase-type plasminogen activator
UVRAG	UV radiation resistance-associated gene
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WT	wild type
Y	tyrosine

Chapter 1

Introduction

1 Introduction

1.1 Protein tyrosine kinases

Protein kinases are enzymes that transfer phosphate groups from adenosine-5'-triphosphate (ATP) molecules to the side chains of specific amino acids present on target proteins. The result of this process is phosphorylation and this alters the biological properties of the target protein. According to which type of amino acid residue that is phosphorylated, protein kinases can be divided into either tyrosine or serine/threonine kinases. However, some kinases may display a dual specificity for both tyrosine and serine/threonine residues.

Based on their topology and localisation, protein tyrosine kinases can be further divided into two families: receptor tyrosine kinases (RTKs) and non-receptor protein tyrosine kinases (NRTKs) [reviewed in (Neet and Hunter, 1996; Ullrich and Schlessinger, 1990)].

Approximately 90 tyrosine kinase genes have been identified within the human genome, 58 of which are RTKs and the remaining 32 encode NRTKs [reviewed in (Robinson et al., 2000)].

1.1.1 Receptor tyrosine kinases

RTKs are transmembrane proteins that are responsible for signal transduction across the plasma membrane. They enable cells to respond to changes in their environment. Through phosphorylation RTKs regulate various intracellular signaling pathways that control cellular processes, such as proliferation, differentiation and apoptosis. They also have a critical role in development and disease. Based on the kinase domain sequence they are grouped into 20 subfamilies, and well known examples

include the epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), and Tyro-3, Axl, and Mer (TAM) RTKs [reviewed in (Lemmon and Schlessinger, 2010)].

The majority of RTKs are single transmembrane receptors and possess a similar molecular architecture with an extracellular N-terminal region that binds to ligands, a single hydrophobic membrane-spanning region which plays a key role in receptor dimerisation, and a cytoplasmic portion that contains the tyrosine kinase activity [reviewed in (Maruyama, 2014)].

The extracellular portion of RTKs usually has a highly variable, multi-domain structure, with the most common modules being immunoglobulin-like repeats, fibronectin-type III repeats and cysteine rich domains. The variability of these extracellular domains provides the specificity for ligand binding [reviewed in (Hubbard and Miller, 2007)]. The intracellular domains of RTKs are more structurally similar and contain highly conserved tyrosine kinase domains. The kinase domains are frequently flanked by juxtamembrane domains and a carboxy-terminal (C-terminal) tail, which are important for kinase regulation and downstream signaling [reviewed in (Lemmon and Schlessinger, 2010)].

The mechanism of RTK activation has been thoroughly characterised through structural and biochemical studies. It is now accepted that activation of RTKs occurs by oligomerisation of its ligand to the extracellular domain, which triggers the formation of functional dimeric receptors [reviewed in (Schlessinger, 2000)]. Dimerisation may occur between identical receptors, between isoforms of the same receptor or between members of the same subfamily. Dimerisation of the receptors

brings together the two kinase domains, which then phosphorylate a tyrosine residue present in the activation loop of each other [reviewed in (Schlessinger, 2000)]. Phosphorylation increases the catalytic activity and leads to the phosphorylation of other tyrosine residues present in the molecule, thereby forming docking sites for several downstream signal transduction proteins with Src homology (SH2) or phosphotyrosine-binding (PTB) domains (Figure 1) [reviewed in (Lemmon and Schlessinger, 2010; Schlessinger and Lemmon, 2003)]. These proteins are, in turn, phosphorylated and activated by the growth factor receptors, hence leading to the activation of a series of signaling pathways that mediate their biological effects. It has been shown that heterodimerisation may result in different autophosphorylation states compared to homodimerisation, therefore contributing to the diversity of signaling (Olayioye et al., 1998).

Signaling processes linked with RTKs are often altered in tumour cells, and are associated with their more aggressive cellular growth or increased invasiveness. Therefore, RTKs have become of great interest as targets for anti-cancer therapies. There are two main categories of RTK inhibitors currently being developed: small-molecule inhibitors that target the ATP-binding site of the intracellular tyrosine kinase domain and monoclonal antibodies (mAbs) that are directed against the extracellular domain of RTKs [reviewed in (Fauvel and Yasri, 2014; Shawver et al., 2002; Zwick et al., 2002)]. One of the successful small-molecule inhibitor that is approved for patients with advanced non-small cell lung cancer (NSCLC) is Gefitinib (ZD1839, Iressa). Gefitinib inhibits the EGFR kinase activity by binding the ATP-binding site within the catalytic domain. Most patients with NSCLC that respond to Gefitinib treatment carry somatic mutations within the ATP-binding

pocket of EGFR. It has been shown that Gefitinib may mediate anti-tumour activity by inhibition of anti-apoptotic signals transduced by the mutant receptor (Sordella et al., 2004).

Anti-RTK mAbs might work by preventing ligand-receptor interaction. They bind either to the ligand or the receptor. In the case of human breast cancers, cells expressing a constitutively active mutant form of the HER2 receptor enable the uncontrolled proliferation of cancer cells, even in the absence of EGF, which is required for the proliferation of normal cells. Mutations of HER2 gene occur mainly in the kinase domain of the receptor and result in conformational change and increasing kinase activity (Bose et al., 2013). Trastuzumab (herceptin), the humanized monoclonal antibody that targets HER2, benefits patients with early and locally advanced breast cancer (Moja et al., 2012). More recently, it is used for the treatment of metastatic disease as well. One of the mechanisms by which trastuzumab inhibits tumour growth is mediated through inhibition of the PI3K/Akt pathway. Trastuzumab binds to HER2 and blocks Src signalling and therefore increases the phosphatase and ten-sin homolog (PTEN) level and activity. PTEN is a phosphatase that negatively regulates Akt activity. Suppression of Akt signaling results in reduction in tumour cell growth and survival (Nagata et al., 2004). In addition, trastuzumab inhibits HER2 cleavage and generation of the active truncated membrane-bound fragment p95HER2 (Molina et al., 2001). Moreover, antibody dependent cellular cytotoxicity has been suggested as a possible mechanism of action of trastuzumab (Petricevic et al., 2013). Despite the responsiveness to this inhibitor, the majority of patients who initially respond to trastuzumab develop resistance within a year of treatment. Some of the mechanisms of trastuzumab resistance are

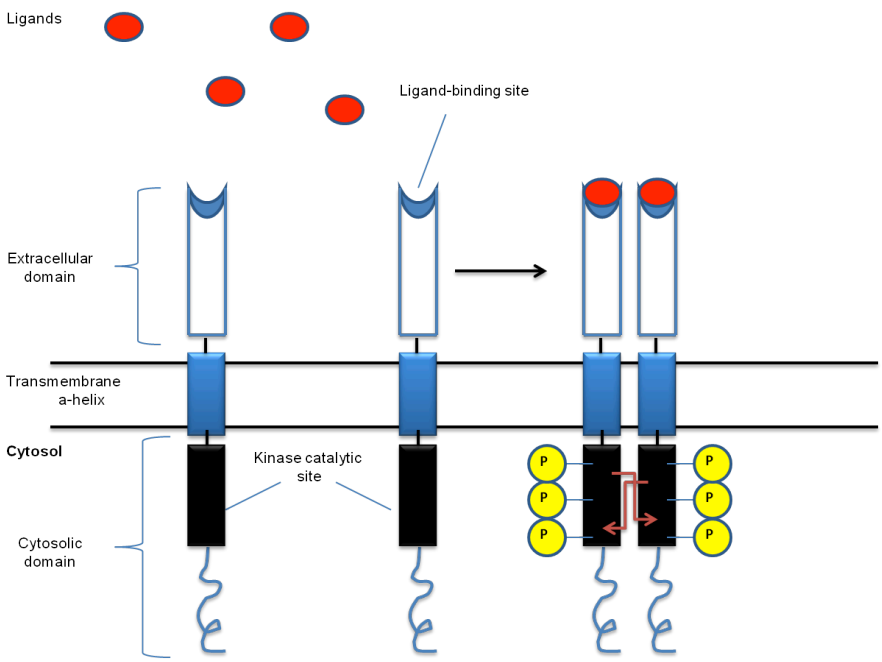


Figure 1.1 Activation of a receptor tyrosine kinase through phosphorylation

The receptor tyrosine kinase protein contains an extracellular domain that binds to ligand (in red), single membrane spanning region (in blue) and cytosolic domain that contains kinase catalytic site (in black). In the absence of a ligand, the tyrosine kinase domain is in a low-activity state. Ligand – mediated dimerisation leading to tyrosine kinase activation and tyrosine phosphorylation. After phosphorylation kinase domains become fully active and phosphotyrosines serve as docking sites for SH2 domain - containing proteins.

prevention of trastuzumab binding to HER2 receptor (Price-Schiavi et al., 2002), the upregulation of HER2 downstream signaling pathways (Pandolfi, 2004), signaling through alternate pathways as IGF-1R, c-Met (Lu et al., 2004; Shattuck et al., 2008), and failure to trigger an immune-mediated destruction of tumour cells [reviewed in (Pohlmann et al., 2009)].

1.1.2 Non-receptor protein tyrosine kinases

NRTKs typically contain a tyrosine kinase domain, and protein-protein, protein-lipid and other interaction domains. Protein-protein interaction domains include SH2 and SH3 domains [reviewed in (Hubbard and Till)]. An SH2 domain (~100 residues) enables the protein carrying it to associate with another protein's phosphotyrosine residue in a sequence specific manner, thus forming a physical complex between these two proteins. The SH3 domain (~60 residues) binds specifically to proline-rich sequence domains present on target proteins. Protein-lipid interaction domains, such as pleckstrin homology (PH) domains and FERM domains, can bind to phosphorylated phosphatidylinositol lipids that can lead to the recruitment of these proteins to the membrane. These are only a few examples of a very large number of modular protein/lipid interaction domains [reviewed in (Balla, 2005)].

Activation of NRTKs occurs through the phosphorylation of tyrosine residues present within the activation loop and results in increased enzymatic activity. However, tyrosine phosphorylation can also negatively regulate kinase activity, for example the phosphorylation of SrcY527 holds the molecule in a closed inactive conformation [reviewed in (Boggon and Eck, 2004)].

The family of NRTKs include, among others, the Src family kinases, the family of Janus kinases (JAKs), focal adhesion kinases (FAK) and the Abelson (ABL) family kinases. NRTKs regulate multiple cellular processes, including cell proliferation, survival, adhesion and migration that are crucial for tumour progression and metastasis; therefore, they become useful targets for anti-cancer therapy. One of the first successful small-molecule inhibitors that are approved by the US Food and Drug Administration (FDA) is Gleevec (Imatinib mesylate) (Zhang et al., 2009). Gleevec inactivates the kinase activity of the BCR-ABL fusion protein in Chronic Myeloid Leukemia (CML). Patients with CML treated with Gleevec often experience relapse due to drug-resistance. The main reason to this is point mutations within the BCR-ABL kinase domain (Shah et al., 2002).

1.2 Src kinase family members

The Src kinase family is a family of NRTKs that includes nine members: Src, Lck, Hck, Fyn, Yes, Lyn, Blk, Fgr and Yrk. These are all regulatory proteins, which play a key role in cell differentiation, motility, proliferation and cell survival [reviewed in (Boggon and Eck, 2004; Engen et al., 2008)]. All the members of this family share the same domain organisation structure, with modular SH3, SH2 and tyrosine kinase domains followed by a C-terminal regulatory tail. All Src-family members also contain an N-terminal membrane-targeting region, which is always myristoylated, and in some Src-family members may be additionally palmitoylated. In addition, each family member also has 50-80 unique amino acids in their N-termini, which are involved in interactions with specific receptors or proteins [reviewed in (Boggon and Eck, 2004).

Each Src-family member has a different pattern of cellular expression and localisation. While some family members are ubiquitously expressed, such as Yes, c-Src and Fyn, others demonstrate a restricted distribution. It has been shown that Lck plays a critical role in T-cell signalling, however Hck is expressed primarily in monocytic and granulocytic hematopoietic cells [reviewed in (Korade-Mirnics and Corey, 2000; Thomas and Brugge, 1997)].

1.2.1 c-Src

In 1911, Peyton Rous discovered that a cell-free extract of a chicken tumour was able to induce oncogenesis in other healthy chickens. The 'filterable agent', later called Rous sarcoma virus, could cause cancer was at first controversial, but in 1970 it was proven that the malignant properties of RSV could be attributed to a viral gene, *v-Src*. Subsequent research discovered a cellular equivalent of *v-Src*, termed *cellular Src* (*c-Src*) (Stehelin et al., 1976). This gene was found to be conserved within the vertebrate genome thereby indicating that the *v-Src* gene had been incorporated into the viral genome through recombination. Upon the characterisation of the v-Src and c-Src proteins, Src was shown to be a NRTK involved in signal transduction.

1.2.2 Structure of Src

The Src protein is 60kDa and is widely expressed in mammalian cells, with particularly high levels present in the brain, platelets and osteoclasts.

The structure of Src and its regulation are well understood as a result of both structural and mutational studies. Src consists of four domains: a unique amino-terminal domain containing a myristoylation segment; two SH domains, SH2 and SH3; a protein-tyrosine kinase domain, containing tyrosine Y416 (Y419, human); and a carboxy-terminal tail containing a negative regulatory tyrosine residue Y527

(Y530, human), which interacts with the SH2 domain following C-terminal Src kinase (Csk)-mediated phosphorylation. In v-Src Y527 is mutated, rendering it constitutively activated (Figure 2) [reviewed in (Engen et al., 2008)].

N-terminal myristoylation is required for both membrane localisation and biological activity of Src and it is crucial for the transformation of oncogenic Src mutants as mutations in the N-terminal domain reduce the transforming potential of v-Src. The SH2 domain of Src contains approximately 100 amino acids and it is able to recognise and bind to sequences containing a phosphorylated tyrosine residue, whilst the SH3 domain binds to proline-rich (PXXP) segments of target proteins [reviewed in (Boggon and Eck, 2004; Engen et al., 2008)]. The tyrosine kinase domain has a two-lobe structure that is also common to other protein kinases.

1.2.3 Src activation

In normal cells Src activity is tightly regulated, 90-95% of Src is in an inactive state and becomes transiently activated during specific cellular events. Several mechanisms of activation of Src have been proposed [reviewed in (Bjorge et al., 2000; Ingley, 2008; Okada, 2012)].

Phosphorylation of Y527 is one of the mechanisms for the regulation of Src activity. When Y527 is phosphorylated, the molecule adopts a folded, inactive configuration that is mediated by an intra-molecular interaction between the phosphorylated Y527 and the SH2 domain of Src (Roussel et al., 1991). Concurrently, the SH3 domain binds to the linker region that connects the SH2 and kinase domains (Xu et al., 1997). In the folded conformation, neither the SH2 nor the SH3 domain is accessible to the ligands. Subsequent dephosphorylation of Y^{527} by tyrosine phosphatases, such as protein tyrosine phosphatase PTP1α and PTP-1B, disrupts the intra-molecular

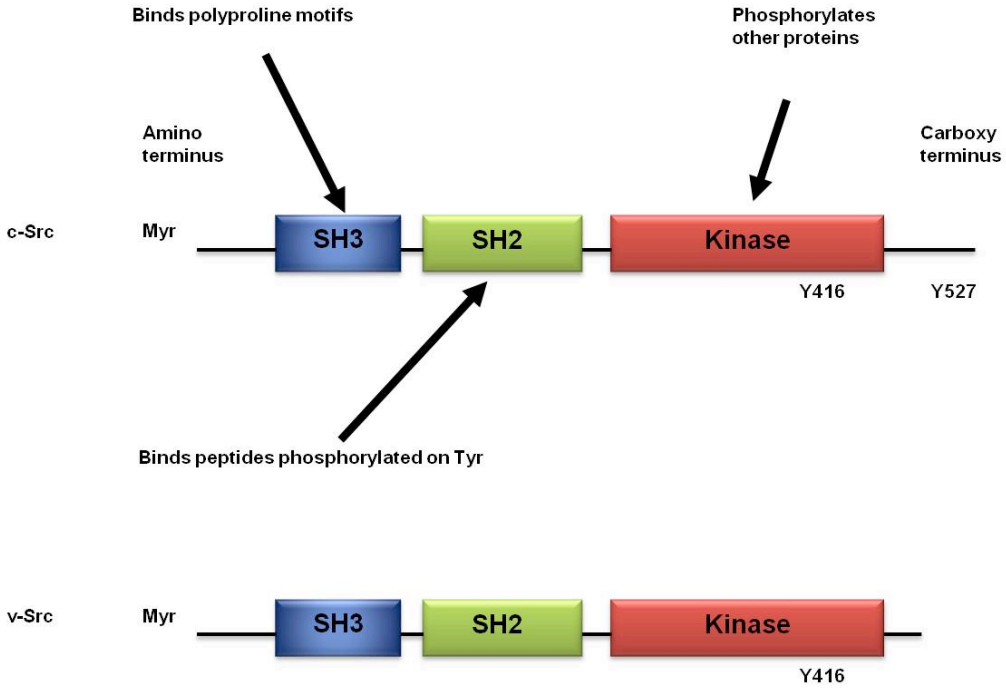


Figure 1.2 Src domain structures of cellular (c-Src) and viral (v-Src)

The Src molecule is composed of a unique amino-terminal domain containing a myristoylation segment, SH2 and SH3 domains, a kinase domain with Y416 and a carboxy-terminal negative regulatory domain, containing Y527, which is absent in v-Src.

interactions of the Src tyrosine kinase and results in an 'open' active conformation. For example, over-expression of PTPα in rat embryo fibroblasts results in a transformed phenotype, a significant reduction in the Y^{527} phosphorylation level and increase in c-Src kinase activity (Zheng et al., 1992). Moreover, increased expression of PTP1B activates Src and induces a Src-dependent transformed phenotype in human breast epithelial cells (Arias-Romero et al., 2009). Full activation of Src also requires the phosphorylation of Y^{416}, which is located in the kinase domain and this is achieved through autophosphorylation. Phosphorylated Y^{416} may stabilise the kinase domain in an active conformation (Martin, 1970). Once Src unfolds it becomes available for interacting with several molecules involved in intracellular signaling. In addition, Src activity can be regulated through its interaction with binding partners, such as FAK. FAK binds to SH2 domain of Src, disturbs the intra-molecular interactions and results in the activation of Src (Schaller et al., 1994). Finally, Src activity can be regulated by ubiquitin-dependant degradation of active Src (Harris et al., 1999).

Src activation can be initiated by several cellular mechanisms including growth factor receptors (PDGFR), G-protein coupled receptors and integrins (reviewed in (Bjorge et al., 2000; Ingley, 2008; Okada, 2012)]. Once activated Src signals to the nucleus through a variety of downstream signaling molecules, including STAT3 (Cirri et al., 1997; Turkson et al., 1998), Ras (Smith et al., 1986), and PI3K (Sugimoto et al., 1984).

1.2.4 Function of Src

Src tyrosine kinase has multiple biological functions, including proliferation, differentiation, survival, the cell cycle, cell adhesion, motility and actin dynamics

[reviewed in (Frame, 2002; Parsons and Parsons, 2004)]. For example, Src tyrosine kinase is involved in the initiation of DNA synthesis (Broome and Hunter, 1997), the regulation of cell cycle transition G2/M (Taylor and Shalloway, 1996), and in the control of Myc expression by the platelet derived growth factor (PDGF) receptor (Broome and Hunter, 1997). In addition, Src tyrosine kinase activity is also required for focal adhesions turnover in order for cell migration to occur (Westhoff et al., 2004).

To date, nearly 200 Src binding partners have been identified including numerous important signaling proteins, such as FAK (Xing et al., 1994), which is a key binding partner and substrate in many of the functions of Src.

1.2.5 Src and cancer

Multiple studies have supported a role of Src tyrosine kinase in the development and progression of human cancers including breast, colon and pancreatic cancers, where Src kinase activity and protein levels are elevated [reviewed in (Chen et al., 2014; Irby and Yeatman, 2000; Sen and Johnson, 2011)]. These levels appear to increase with the stage of disease. Several mechanisms responsible for activation of Src kinase in cancer have been proposed [reviewed in (Irby et al., 1999)]: 1) activation by receptor tyrosine kinases, 2) activation through posttranslational modification, such as phosphorylation of Y527 by Csk, 3) activating mutations in a *Src* gene. For example, mutation in Src at codon 531 has been reported to play a role in Src activation in a subset of advanced human colon cancers (Irby et al., 1999). However, Src kinase is rarely mutated in human cancers.

It has been shown that Src is involved in several aspects of tumour progression, including proliferation (Wu et al., 2013), invasion (De Luca et al., 2014), metastasis

(Chan et al., 2012; Zhang et al., 2012), and survival (Leung et al., 2009). Therefore, it is not surprising that Src become an important therapeutic target for cancer therapy. There are several Src kinase inhibitors that are undergoing in clinical trials, such as dasatinib, bosutinib and Saracatinib.

1.3 Focal adhesion kinase

1.3.1 FAK discovery

FAK is a 125 kDa NRTK which was first isolated in 1992 through the co-immunoprecipitation of tyrosine phosphorylated proteins from chicken embryo cells transformed with Rous sarcoma virus, *v-Src* (Schaller et al., 1992), and from growing cultures of BALB/c3T3 fibroblasts (Hanks et al., 1992). Both laboratories demonstrated that FAK localised to focal adhesions, and sequence and structural analyses suggested that it may represent a new family of protein tyrosine kinases. After years of extensive research, FAK has emerged as a key signaling molecule that plays an important role in transducing signals downstream of integrins and growth factor receptors. It has been shown that at focal adhesions, FAK functions as an adapter protein to recruit other focal adhesion proteins and their regulators. FAK is also involved in the regulation of a number of cellular processes, including cell spreading, migration, invasion, and proliferation [example references are (Brown et al., 2005; Owen et al., 1999; Schlaepfer and Mitra, 2004; Wang et al., 2000)].

1.3.2 Expression of the gene encoding FAK

The human *fak* gene (previously known as *ptk2*) has been mapped to chromosome 8q24.3 (Fiedorek and Kay, 1995) and encodes a cytoplasmic protein tyrosine kinase which is 1052 amino acids in length (Whitney et al., 1993). The human FAK protein

shares more than 95% amino acid identity between both chicken and mouse FAK (Whitney et el., 1993), showing that it is well conserved during evolution.

FAK is expressed ubiquitously and is detected in all cell types, examined so far mesenchymal cells, neuronal cells, platelets, monocytes and lymphocytes (Abedi and Zachary, 1995).

1.3.3 FAK structure and function

FAK contains three main domains: an N-terminal FERM (band 4.1, ezrin, radixin, moesin homology) domain, a central kinase domain, and a C-terminal domain containing the focal adhesion targeting (FAT) sequence and two proline rich regions (Figure 3). FAK is now believed to regulate a variety of cellular processes including cell migration, proliferation, survival and motility [reviewed in (Hall et al., 2011)].

1.3.4 FAK isoforms

Multiple FAK isoforms occur as a result of alternative splicing or through the use of alternative promoters. For example, fibroblasts express an amino-terminally truncated form of FAK, known as FAK related non-kinase (FRNK) (Schaller et al., 1993). FRNK mRNA is generated from an alternative promoter located within an intron downstream of the catalytic domain (Schaller et al., 1993) and functions as a physiological dominant negative inhibitor of integrin dependent FAK activity and downstream signaling (Richardson and Parsons, 1996), therefore exogenous expression of FRNK has been used as a dominant-negative mutant to inhibit FAK signaling in many studies. It has been proposed that FRNK inhibits FAK either by competitive displacement of FAK from focal adhesions or competing with FAK for specific binding partners and preventing downstream signal transduction (Heidkamp et al., 2002).

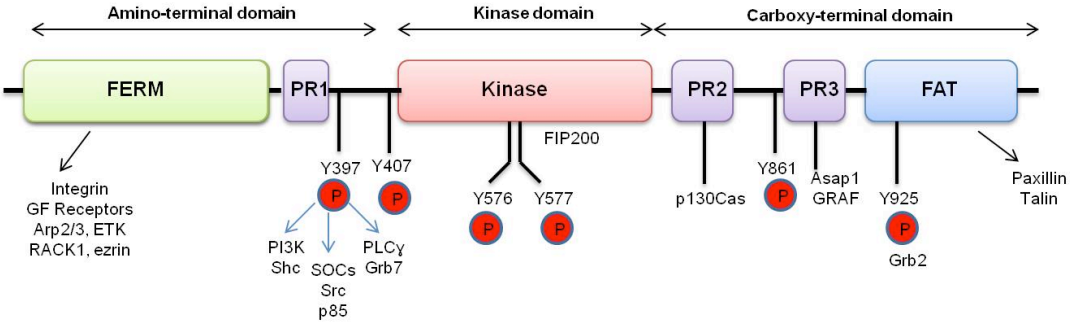


Figure 1.3 FAK domain structure and phosphorylation sites

FAK consists of a FERM domain, a kinase domain and a carboxy-terminal domain containing the FAT sequence, and two proline rich regions that bind to SH3-domains of target proteins. FAK has multiple tyrosine phosphorylation sites, including Y397, Y407, Y576/577, Y861 and Y925.

The major form of alternative splice variant is known as FAK+, which contains a three amino acid (Pro-Trp-Arg) insertion within the C-terminal domain. It is expressed at very low levels in non-neuronal cells while is highly expressed in the central nervous system (Burgaya and Girault, 1996). Other FAK isoforms (FAK box 6, box7 and box 28) contain alternative insertion of peptides of six, seven or twenty-eight amino acids in length coded by additional exons surrounding the FAK-Y397 site (Burgaya et al., 1997). The presence of additional exons does not prevent the targeting of FAK to adhesion sites; however alternatively spliced FAK isoforms exhibit elevated levels of tyrosine phosphorylation compared to wild type FAK (Messina et al., 2003). These isoforms are mostly found in brain tissues, thereby suggesting that neuronal FAK may have unique properties and/or functions.

1.3.5 FAK family members

A second member of FAK family was identified by four independent groups and termed Pyk2 (Lev et al., 1995), cell adhesion kinase β (Sasaki et al., 1995), related adhesion focal tyrosine kinase (RAFTK) (Avraham et al., 1995) and calcium-dependent tyrosine kinase (CADTK) (Yu et al., 1996). FAK and Pyk2 share around ~45% overall sequence similarity, constituting the kinase domains, regions surrounding the conserved tyrosine phosphorylation sites responsible for binding SH2 domain containing proteins, and two proline-rich regions responsible for binding SH3 domain containing proteins [reviewed in (Schlaepfer et al., 1999)].

Like FAK, Pyk2 contains a central kinase domain flanked by N- and C-terminal non-catalytic regions. It has four tyrosine phosphorylation sites: Y402, Y579/80 and Y881 at analogous positions to that of FAK (Y397, Y576/577 and Y925), and Y402 is the major autophosphorylation site. It also serves as a binding site for the SH2

domain of Src (figure 4) [reviewed in (Avraham et al., 2000; Lipinski and Loftus, 2010)].

Alternative splicing of *pyk2* mRNA transcript generates a truncated variant of Pyk2 with a 42 amino acid deletion within the C-terminal domain (Dikic and Schlessinger, 1998; Xiong et al., 1998), and this is mainly expressed in thymocytes, B cells, monocytes and natural killer cells (Dikic and Schlessinger, 1998).

Despite their structural similarity, Pyk2 and FAK show a number of significant differences. While FAK is expressed in virtually almost all tissues and cell types, expression of Pyk2 appears to be more restricted to cells of hematopoietic lineages and the central nervous system (Avraham et al., 1995; Lev et al., 1995). In addition, despite carrying a FAT domain, Pyk2 is not normally associated with focal adhesions, but instead is mostly found in the perinuclear area of the cell [reviewed in (Schaller, 2010)]. There is also ample evidence to suggest that intracellular Ca^{2+} levels regulate its activation (Guo et al., 2004; Lev et al., 1995). While FAK-/- mice die at approximately day 9 of embryonic development (Ilic et al., 1995), Pyk2 deficient mice remain viable and fertile (Guinamard et al., 2000).

Several reports showed that deletion of FAK could lead to increased expression of Pyk2 to compensate for the loss of FAK (Fan and Guan, 2011; Sieg et al., 1998; Weis et al., 2008). For example, Pyk2 protein expression was upregulated in mammary cancer stem cells (MaCSCs) and metastatic nodules after FAK deletion in mammary tumour cells (MFCKO-MT mice), and its inhibition significantly reduced mammary tumour development and metastasis in this mice (Fan and Guan, 2011).

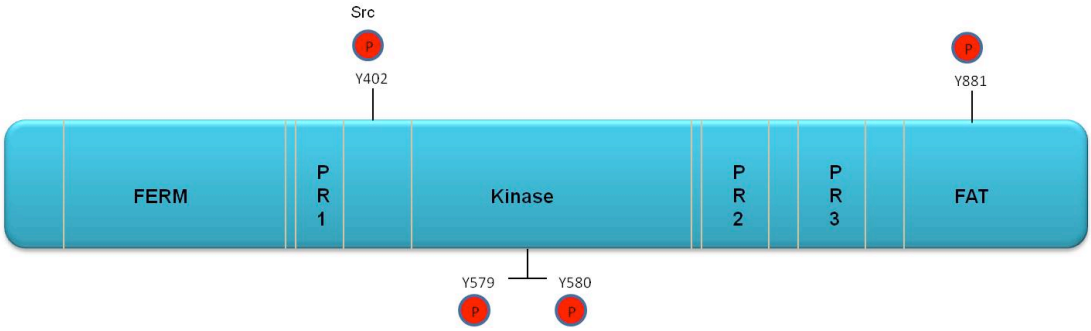


Figure 1.4 Pyk2 domain structure and phosphorylation sites

Pyk2 is composed of amino-terminal FERM, kinase, and carboxy-terminal FAT domains. It also has three proline rich regions that mediate interaction with SH3-domain containing proteins. Pyk2 has four tyrosine phosphorylation sites. Y402 is an autophosphorylation site which serves as a docking site for SH2 domain of Src. Adapted from (Lipinski and Loftus, 2010).

1.3.6 FAK phosphorylation

FAK has multiple phosphorylation sites (Y397, Y407, Y576, Y577, Y861 and Y925), among which, Y397 is the autophosphorylation site of FAK and is involved in its initial activation following integrin engagement (Calalb et al., 1995; Schaller et al., 1994; Schlaepfer and Hunter, 1996). Moreover, phosphorylated Y397 is required for interactions with a number of SH2 domain containing proteins, including the Src family of protein tyrosine kinases (Xing et al., 1994), the regulatory subunit of phosphoinositide (PI) 3-Kinase, p85 (Chen et al., 1996), the Src homologous and collagen-like protein (Shc) (Barberis et al., 2000), the growth factor receptor bound protein 7 (Grb7) (Han and Guan, 1999), and phospholipase C gamma (PLCγ) (Zhang et al., 1999).

Recruitment of Src to phosphorylated FAK-Y397 is important event as it promotes Src dependent phosphorylation of some of the remaining FAK tyrosine residues [reviewed in (Schlaepfer et al., 1999)]. Phosphorylation of Y576 and Y577, which are located in the activation loop of the kinase domain, is necessary for full catalytic activity, and mutation of these residues reduces FAK catalytic activity (Calalb et al., 1995). However, mutagenesis studies demonstrated that FAK-Y397 is also important for its maximal kinase activity and downstream signaling (Calalb et al., 1995). Moreover, it has been demonstrated that FAK-Y576/577 may regulate trans-phosphorylation of FAK on Y397 (Leu and Maa, 2002). Src can also phosphorylate Y861 and Y925 located in the C-terminus of FAK. Phosphorylation of Y861 promotes the binding of FAK to the cytoplasmic tails of integrins, which is necessary for VEGF stimulated migration (Eliceiri et al., 2002). It is also thought that phosphorylated Y861 mediates the anti-apoptotic activity of FAK (Abu-Ghazaleh et

al., 2001). Phosphorylated Y925 creates binding sites for Grb2-SH2 (Schlaepfer et al., 1994) and this provides a mechanism of activation for the Ras/mitogen-activated protein kinase (MAPK) pathway (Schlaepfer et al., 1994; Schlaepfer et al., 1999). In addition, Grb2 binding to Y925 also recruits dynamin to focal adhesions, which is important in their disassembly (Ezratty et al., 2005). Therefore, Src dependent phosphorylation of FAK plays an important role in multiple signaling pathways downstream of integrins and growth factor receptors.

At least four different serine phosphorylation sites (S732, S840, S843 and S910) have been recognised within the C-terminal domain of FAK (Ma et al., 2001). Although the function of these serine residues has been less well studied, there is evidence, which implies that the phosphorylation of these serine residues is associated with FAK inactivation, such as during mitosis (Ma et al., 2001).

1.3.7 The FERM domain of FAK

The FERM domain is named after the four proteins it was originally identified in, band 4.1, ezrin, radixin and moesin. FERM domains are found in many cytoskeletal proteins as well as in signaling proteins and multiple tyrosine phosphatases, and they serve as a link between the actin cytoskeleton and the plasma membrane [reviewed in (Girault et al., 1999)]. In general, FERM domains have been shown to mediate both intra-molecular and inter-molecular protein-protein interactions (Dunty et al., 2004; Girault et al., 1999). FERM domains are constructed of three sub-domains (F1, F2 and F3) that adopt a clover-leaf structure and share structural homology with ubiquitin, acyl-CoA-binding protein and pleckstrin homology-phosphotyrosine binding (PH-PTB) domains respectively [(Ceccarelli et al., 2006), reviewed in (Frame et al., 2010)].

The FAK-FERM domain associates with several target proteins, including the cytoplasmic tail of β1 integrin (Chen et al., 2000), growth factor receptors such as EGFR (Sieg et al., 2000), the epithelial and endothelial tyrosine kinase (ETK) (Chen et al., 2001), the actin related protein 3 (Arp3) subunit of the Arp2/3 complex (Serrels et al., 2007), the hepatocyte growth factor receptor c-Met (Chen and Chen, 2006), the EphA2 receptor tyrosine kinase (Carter et al., 2002), and the scaffolding protein receptor for activated C kinase 1 (Rack1) (Serrels et al., 2010). For example, FAK directly binds to Met through its F2 sub-domain and promotes HGF-induced cell invasion (Chen and Chen, 2006). Moreover, FAK interactions have been shown to play a role in the regulation of multiple cellular processes, such as the formation of early adhesion structures required for cell spreading, and for directional sensing required during cell polarity and invasion (Serrels et al., 2010).

In addition to protein binding, there are indications that the FAK FERM domain is also involved in the regulation of FAK activity and phosphorylation, as deletion of the amino-terminal domain of FAK leads to an increase in kinase activity (Cooper et al., 2003). There is also evidence demonstrating that deletion of the first 375 amino acids of FAK leads to elevated FAK phosphorylation even when Y397 is mutated to phenylalanine (Cohen and Guan, 2005). This finding suggests that under certain conditions FAK phosphorylation may be Src independent.

Structural and functional studies suggested that FAK activity is regulated via intra-molecular interactions (Dunty et al., 2004; Lietha et al., 2007). The direct interaction between the N-terminal FERM and kinase domains masks both the linker region containing FAK-Y397 and the activation loop within the kinase domain (Lietha et al., 2007), therefore displacement of the FERM domain is an important initial step in

FAK activation. Binding of FERM domain interacting protein partners, such as C-Met, leads to FAK activation (Chen and Chen, 2006), whereby the FERM domain is released and FAK-Y397 is autophosphorylated. This is followed by Src binding to the autophosphorylation site of FAK in order to facilitate phosphorylation of the remaining five tyrosine residues (Y407, Y576, Y577, Y861 and Y925) [reviewed in (Mitra et al., 2005)], thereby resulting in a fully activated FAK molecule.

Although FERM domains are known to bind cytoskeleton- or membrane-linked proteins, it seems that the FAK FERM domain has yet another function. Under conditions of cellular stress, the FERM domain can facilitate nuclear translocation of FAK (Lim et al., 2008). This nuclear targeting of FAK is due to the presence of a nuclear localisation signal (NLS) sequence in the FERM domain of FAK (Lim et al., 2008). Nuclear FAK has been shown to promote cell proliferation and survival through FERM-enhanced degradation of the p53 protein (Lim et al., 2008).

1.3.8 The kinase domain of FAK

This domain shares a high degree of sequence similarities with other protein tyrosine kinases; however, unlike some other NRTKs, FAK does not contain SH2 and SH3 domains that are involved in protein-protein interactions (Schaller et al., 1992).

Although recent studies show that the FAK FERM domain has a prominent role in the intramolecular regulation of FAK activity by binding to the kinase domain (Cooper et al., 2003; Lietha et al., 2007), previous studies have indicated that FAK kinase activity can also be regulated in several other ways: by direct binding of the negative regulator FAK-interacting protein of 200kDa (FIP200) to the kinase domain (Abbi et al., 2002); by interactions with SOCS proteins, such as SOCS-3 and SOCS-1, which target FAK for poly-ubiquitination and degradation (Liu et al., 2003), by

competition with FRNKs for focal adhesion binding sites (Nolan et al., 1999), and by PTPs such as PTPα which act upstream to regulate FAK-Y397 phosphorylation in response to integrin stimulation (Zeng et al., 2003).

Moreover, it has been shown that FAK activity is regulated through its interaction with lipids such as phosphatidylinositol 4,5-biphosphate (PIP2) binds to the FAK FERM domain and triggers conformational changes in FAK (Cai et al., 2008).

1.3.9 The C-terminal domain of FAK

The C-terminal domain of FAK includes the focal adhesion targeting (FAT) sequence and two proline-rich regions located between the FAT sequence and the kinase domain. The importance of the FAT domain comes from the fact that it is both necessary and sufficient for targeting of FAK to focal adhesions, which was demonstrated by deletion experiments (Hildebrand et al., 1993). Deletion of amino-terminus of FAK had no effect on the localisation of FAK, while the deletion of FAT domain abolished its focal adhesion localisation. Moreover, when the FAT domain was fused to cytosolic protein, was capable of mediating focal adhesion localisation (Hildebrand et al., 1993; Hildebrand et al., 1995).

The FAT domain has been shown to bind to two focal adhesion associated proteins, paxillin and talin (Chen et al., 1995; Hildebrand et al., 1995). Paxillin is one of the first proteins recruited to focal adhesions as they are formed, and there are two binding sites for paxillin within the FAT domain of FAK, known as paxillin binding site 1 (PBS1) and 2 (PBS2) (Hildebrand et al., 1995). FAK is recruited to focal adhesions through its interaction with paxillin, and the disruption of this interaction leads to mislocalisation of FAK (Tachibana et al., 1995). This suggests that binding of paxillin to FAK is necessary for proper localisation. This is further supported by

data showing that in the absence of paxillin there is inefficient localisation of FAK to focal adhesions (Hagel et al., 2002). However, the requirement for paxillin in FAK adhesion recruitment is controversial, because other studies have shown that FAK mutants that are unable to bind paxillin can still correctly localise to focal adhesions (Hildebrand et al., 1995; Schaller et al., 1993). Therefore, the role of paxillin binding to FAK in focal adhesion localisation remains unclear.

Talin is an adaptor protein, which was initially discovered as a protein highly enriched at focal adhesion site (Burridge and Connell, 1983). It has been demonstrated that talin plays an essential role in 'inside-out' signaling required for integrin activation. For example, Talin head domain binds to the integrin β-tail and induces the conformational changes in the integrin ectodomain (Ye et al., 2010a). Talin contains an N-terminal head (talin-H) followed by a rod domain (talin-R). Talin-H consists of four sub-domains: F0, F1, F2 and F3, from which F2 and F3 are involved in binding to FAK [reviewed in (Das et al., 2014)]. Although it was widely believed that talin is required for FAK recruitment and activation at focal adhesions (Chen et al., 1995; Zhang et al., 2008), Lawson et al. have recently proposed an alternative model, which suggests that it is FAK that recruits talin to focal adhesions (Lawson et al., 2012). When talin binding site on FAK is mutated, talin fails to localise to nascent adhesions, however this mutation does not disrupt integrin-mediated FAK recruitment and activation (Lawson et al., 2012).

The proline-rich regions of FAK mediate interactions with SH3 domain containing proteins, such as p130Cas (Polte and Hanks, 1995), c-Cbl associated protein (CAP) (Ribon et al., 1998), GTPase regulator associated with FAK (GRAF) (Hildebrand et

al., 1996) and Arf-GAP containing a SH3 domain, ankyrin repeats, and PH domain (ASAP1) (Liu et al., 2002).

P130Cas is an adaptor protein that plays a central role in actin cytoskeletal reorganization. Its interaction with proline-rich region of FAK spanning amino acids 712-718 was first identified using a two-hybrid screen, which was later confirmed by co-immunoprecipitation (co-IP) experiments in mouse fibroblasts (Polte and Hanks, 1995). CAP is an adaptor protein that plays a key role in the regulation of the actin cytoskeleton and cell adhesion. It consists of N-terminal sorbin homology (SoHo) domain and three C-terminal SH3 domains [reviewed in (Tomasovic et al., 2012)]. GST pull-down experiments showed that CAP interacts with FAK through the third SH3 domain (Ribon et al., 1998). GRAF bids to proline-rich regions of FAK through SH3 domain interaction and preferentially stimulates RhoA and cdc42 activity (Hildebrand et al., 1996). ASAP1 interacts with proline-rich motif of FAK via its C-terminal SH3 domain. It has been shown that over-expression of ASAP1 inhibits cell spreading and the localisation of paxillin and FAK to focal adhesions (Liu et al., 2002).

1.4 FAK regulation of cellular functions

FAK plays an important role in the regulation of several biological processes, including cell proliferation, cell migration, cell survival and apoptosis [reviewed in (Abbi and Guan, 2002)]. In this section the evidence associating FAK with these biological processes will be discussed, with an emphasis on the relevance to cancer.

1.4.1 FAK – proliferation and cell cycle

Numerous studies have demonstrated a regulatory role of FAK in cell cycle progression. For example, inhibition of FAK through the expression of exogenous dominant negative FRNK, or by microinjection of anti-FAK monoclonal antibodies, causes cell cycle arrests at G1/S phase boundary (Gilmore and Romer, 1996; Hungerford et al., 1996). Early experiments suggested that FAK may regulate cell cycle progression through regulating transcription of cyclin D1 (Zhao et al., 2003; Zhao et al., 2001).

Overexpression of FAK in smooth muscle cells increased expression of S-phase kinase-associated protein 2 (Skp2) and increased proliferation (Bond et al., 2004). FAK stabilises Skp2 protein that recognises phosphorylated p27Kip1, and facilitates its ubiquitination and degradation. p27Kip1 is a negative regulator of the cell cycle [reviewed in (Sgambato et al., 2000)].

In addition, FAK is necessary for EGF-mediated proliferation (Flinder et al., 2013). In this study, the authors showed that hepatocytes transfected with various FAK mutants (FAK-Y397F, FAK-Y861F and FAK-Y925F) demonstrated markedly lower level of Cdk2 expression in response to EGF compared to cells expressing FAK-WT, suggesting that Cdk2 protein expression is regulated by FAK.

Although, FAK signaling plays a role in the regulation of cell cycle progression, it does not appear to be required. For example, FAK-/- cell and cells treated with FAK RNAi still proliferate (Ilic et al., 1995).

1.4.2 FAK and cell migration

Cell migration is central to many physiological and pathological processes, including embryonic development, wound healing, inflammation and tumour metastasis. Cell migration involves a continuous formation, maturation and disassembly of focal adhesions that are formed the edge of the lamellipodial protrusions in spreading cells [reviewed in (Gardel et al., 2010; Jockusch et al., 1995)]. The function of focal adhesions is to link actin filament bundles to the extracellular matrix (ECM) via integrins, and hence provides the traction required for cell migration. Many proteins are present in focal adhesions that specifically control the actin dynamics required during cell migration, such as adaptor proteins, structural proteins, and cell surface receptors, including integrins and other signaling molecules.

There is ample evidence indicating the positive role of FAK in cell migration via multiple signaling partners. Initial studies in FAK deficient mouse embryo fibroblasts (MEFs) demonstrated that a FAK deficiency led to a reduction in the rate of focal adhesion turnover, and impaired integrin and growth factor induced cell migration [reviewed in (Parsons et al., 2000)]. This defect in cell migration was rescued by reconstitution of FAK-deficient cells with wild type FAK, but not by FAK mutants lacking kinase activity (FAK-KD) or lacking the autophosphorylation site (FAK-Y397F) and thus the ability to bind Src family kinases, demonstrating that the catalytic activity of FAK is required. Further evidence indicates that FAK mutants that cannot bind to p130Cas also fail to induce cell migration (Reiske et al., 1999). Similarly, over-expression of FAK in Chinese hamster ovary fibroblasts led to enhanced migration (Cary et al., 1996), while overexpression of the C-terminal

portion of FAK, functions as a dominant negative FAK and inhibits cell migration (Gilmore and Romer, 1996).

In our laboratory, work showed that FAK, via its FERM domain, could bind directly to, and activate the actin-related protein 2/3 (Arp2/3) complex, thereby stimulating leading edge protrusion (Serrels et al., 2007). Arp2/3 complex binds to the side of an existing actin filaments and stimulates the formation of branched actin filament networks [reviewed in (Campellone and Welch, 2010)].

Furthermore, inhibition of FAK expression in mouse fibroblasts by the FAK siRNA resulted in altered lamellipodia formation (Tilghman et al., 2005). As the lamellipodium is an important structure for the establishment of polarity during migration, this work suggests that, the ability of FAK to control cell migration is achieved through the regulation of cell polarity. Work produced in our laboratory indicates that FAK indeed controls directional cancer cell migration via cell polarity (Serrels et al., 2010). FAK promotes PDE4D5 recruitment to the leading edge of polarising cells via the molecular scaffold RACK1. It has been shown that FAK/RACK1/PDE4D5 complex is necessary for nascent adhesion stabilisation and wound-induced polarisation.

In addition, FAK promotes cell migration through direct modulation of the proteins that are involved in the re-modelling of the actin cytoskeleton, including the Rho subfamily of small GTPases and Neuronal Wiskott-Aldrich syndrome protein (N-WASP) (Ren et al., 2000; Wu et al., 2004). For example, FAK binds to N-WASP and promotes its function by regulating its sub-cellular localisation. N-WASP then activates the Arp2/3 complex to induce actin polymerisation (Wu et al., 2004).

Finally, Grb7 is required for FAK effects on migration (Han and Guan, 1999; Han et al., 2000). Therefore FAK regulates multiple pathways required for efficient cell movement.

1.4.3 FAK and cell invasion

Cell invasion is a complex process that can occur through changes in the surface expression of integrins [reviewed in (Hood and Cheresh, 2002)], the release of matrix metalloproteinases (MMPs) that are involved in the degradation of the ECM [reviewed in (Fingleton, 2006)] and by changes in gene expression in transformed cells (Johnston et al., 2000).

FAK is critical for cell invasion and metastasis through regulating the expression and activation of MMP2, MMP9 and membrane type 1 (MT1)-MMP (Hsia et al., 2003; Wang and McNiven, 2012). Degradation of the ECM by MMPs is mediated by specialised surface protrusions, knows as podosomes (Wu et al., 2005) and invadopodia (Linder, 2007). Invadopodia is also known as podosome type adhesion. Both podosomes and invadopodia contain 'actin-rich core' that is formed by proteins involved in actin nucleation. Although, podosomes and invadopodia share similar overall architecture, there are some differences, such as, in podosomes 'actin-rich core' is surrounded by a ring structure formed integrin receptors, mainly β2 and β3 integrins. However, this organisation is not well defined in invadopodia [reviewed in (Block et al., 2008; Murphy and Courtneidge, 2011)]. It has been shown that FAK promotes the expression of MMP9 and the activation of MMP2 through the Src/p130Cas/Dock180 signaling cascade. This cascade induces activation of Rac and c-Jun NH2-terminal kinase (JNK). Subsequent phosphorylation and activation of c-

Jun led by Rac and JNK activation, mediates the transcription of several genes, including MMP genes (Hsia et al., 2003).

The FAK/PI3K/AKT signaling pathway also contributes to hepatocellular carcinoma (HCC) cell invasion through the regulation of MMP2 and MMP9 (Chen et al., 2013). MMP2 and MMP9 expression and activation, and HCC cell invasion, are abolished by siRNA-mediated FAK knockdown (Chen et al., 2013). Moreover, fibronectin induced lung cancer cell migration and invasion occurs through FAK/PI3K/MMP9/RhoA signaling pathway (Meng et al., 2009). In addition, dominant-negative inhibition of FAK activity by FRNK inhibited v-Src stimulated cell invasion through Matrigel and blocked experimental metastasis in a nude mouse (Hauck et al., 2002). This was associated with decreased MMP2 mRNA levels and MMP2 secretion.

Over-expression of MT1-MMPs has been found in many human tumours and is associated with increased invasion and metastasis [reviewed in (Egeblad and Werb, 2002)]. Although podosomes are the primary site for ECM degradation, it appears that this process could also take place at focal adhesions (Wang and McNiven, 2012). Wang and McNiven (2012) showed that ECM degradation at focal adhesions is MT1-MMP dependent, and that this protease is targeted to focal adhesions by a physical interaction with the FAK-p130Cas complex. They also demonstrated that MT1–FAK–p130Cas complex formation is regulated by Src mediated phosphorylation of Y573 at the cytoplasmic tail of MT1 (Wang and McNiven, 2012). MT1-MMP is a zinc dependent type 1 transmembrane metalloproteinase. Several substrates of MT1-MMPs have been identified, including type 1 collagen, fibronectin, laminin-1 and -5, CD44 and αv integrin (Sato et al., 2005).

Another study suggested that FAK could contribute to cell invasion by regulating the expression of urokinase-type plasminogen activator (uPA). uPA is a serine proteinase that catalyses the conversion of plasminogen to the active proteinase plasmin, which is able to degrade various ECM components and basement membrane proteins, and plays a role in cell migration and tissue remodelling. FAK catalytic activity regulates uPA expression in 4T1 breast carcinoma cells, and thus facilitates metastatic breast cancer progression (Mitra et al., 2006a).

Taken together, FAK appears to regulate cell motility and invasion by distinct pathways; by promoting the dynamic regulation of focal adhesions and peripheral actin structures during migration, as well as by MMP-mediated matrix degradation [reviewed in (Tomar and Schlaepfer, 2009)].

Many cancer cells can exhibit two different modes of invasion, referred to as mesenchymal-like (MMP dependent) and amoeboid-like (MMP independent) mechanisms. Mesenchymal type of invasiveness is driven by small GTPases, mainly by Rac and cdc42, while amoeboid-like invasiveness is promoted by the Rho/ROCK signaling pathway (Nobes and Hall, 1995; Sahai and Marshall, 2003). During mesenchymal-like invasion MMPs are recruited at focal adhesions, where they degrade the ECM and generate path for migration cells. However, amoeboidly migrating cells move without ECM degradation [reviewed in (Pankova et al., 2010)]. There is evidence, that some tumour cells undergo a mesenchymal to amoeboid transition that allows cells to invade without MMPs. For example, when HT1080 fibrosarcoma cells were treated with protease inhibitors, they acquired an amoeboid-like invasive phenotype, which was achieved by modulation of α2β1 integrin receptors. Phosphorylation of FAKY397 was also reduced in these cell lines.

However, it is not clear reduced FAKY397 phosphorylation is a consequence of suppressed integrin activity, or whether it contributes to impaired integrin mediated adhesion of invading cells (Carragher et al., 2006). The precise function of FAK in these modes of invasion remains to be explored; however, since FAK has been implicated in MMP expression, it is likely that FAK is required for mesenchymal-like invasion.

1.4.4 FAK and angiogenesis

Angiogenesis is a process by which new blood vessels are formed from pre-existing capillaries. New blood vessel formation is required for many physiological processes such as embryogenesis, wound healing and organ regeneration. It is also an important factor in several pathological processes, including tumour growth and metastasis.

The balance of pro- and anti-angiogenic signals tightly controls physiological angiogenesis; however there is an imbalance between positive and negative angiogenic factors in tumour cells. Several potential regulators of angiogenesis have been identified, including fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and angiogenin. Tumour associated angiogenesis progress through two phases: the avascular phase, which corresponds to a small liaison of less than 2mm in diameter, and the vascular phase, which ensures tumour growth. These two phases are separated by 'angiogenic switch', which depends on increased production of positive regulators of angiogenesis (Bergers and Benjamin, 2003).

There is a significant body of data supporting the influence of FAK on angiogenesis. A possible role of FAK in angiogenesis was first observed by Polte et al. in 1994,

since during mouse embryonic development, FAK expression was abundant within blood vessels (Polte et al., 1994). Furthermore, increased tyrosine phosphorylation and kinase activity of FAK was found in migrating human umbilical vein endothelial cells (HUVECs) adjacent to wounds (Romer et al., 1994), and a conditional knockout of FAK in mouse endothelial cells also resulted in embryonic lethality due to vascular defects (Shen et al., 2005). It has been shown that the *in vitro* deletion of FAK in mouse endothelial cells causes reduced tubulogenesis, decreased cell survival, proliferation and migration (Shen et al., 2005). In contrast, overexpression of FAK in vascular endothelial cells directly promotes angiogenesis in transgenic mice (Peng et al., 2004). Several studies have also highlighted the importance of FAK activity in mediating tumour angiogenesis (Halder et al., 2006; Halder et al., 2007; Mitra et al., 2006b). For example, it has been shown that FAK regulates VEGF expression and induces an angiogenic switch in cancers (Mitra et al., 2006b), thereby suggesting that FAK kinase inhibitors could be useful anti-angiogenesis agents.

Several anti-angiogenic drugs have been developed some of which are in clinical trials. Some drugs appear to directly target molecules involved in new blood vessel formation (SU5416), while others inhibit proliferation of endothelial cells [reviewed in (Rosen, 2000). SU5416 is a small molecular inhibitor, which targets VEGF receptor. It has been shown that anti-VEGF therapy has significant effects in various human cancers; however some cancer patients, who initially respond, show escape from anti-VEGF treatments (Loges et al., 2010). For example, there is evidence that blockade of VEGF signaling leads to up-regulation of other pro-angiogenic growth factors, such as members of FGF family (Casanovas et al., 2005).

1.4.5 FAK and cell survival

Programmed cell death is a vital cellular homeostatic mechanism characterised by distinct biochemical (fragmentation of genomic DNA) and morphological changes (shrinkage of the cell, membrane blebbing, formation of apoptotic bodies) [reviewed in (Portt et al., 2011)]. The initiation phase of apoptosis involves the recognition of a death stimulus. In general, apoptosis can be triggered by two pathways called intrinsic or extrinsic. The intrinsic pathway is triggered by internal signals such as DNA damage. Two of the pro-apoptotic proteins, Bax and Bak, promote the release of pro-apoptotic factors from the mitochondrial inter-membrane space that leads to the recruitment and activation of caspase 9. The extrinsic pathway involves the binding of death receptors to protein ligands that are expressed on the surface of other cells. This binding activates the receptor that leads to the generation of activated caspase 8 [reviewed in (Tait and Green, 2008)]. Both of pathways culminate in the generation of caspase 3 and 7, which leads to the cleavage of a number of cellular proteins and cell death.

The first evidence that FAK can control cell survival was reported by Frisch et al. (1996), who showed that a membrane targeted, activated form of FAK was able to rescue kidney epithelial cells from suspension-induced cell death, known as anoikis (Frisch et al., 1996). Anoikis is a Greek word and means “without house”, thus they used this word to describe the apoptotic response of cells to the absence of cell-matrix interaction. Their study demonstrated that both FAK-Y397 phosphorylation and the kinase activity of FAK were required for cell survival. In addition, increased FAK expression has also been linked to the suppression of apoptosis in the HL60 leukaemia cell line and again, phosphorylation of FAK, as well as its kinase activity,

was required (Sonoda et al., 2000). Furthermore, inhibition of FAK by antisense oligonucleotide (Wu et al., 2006) or the injection of monoclonal FAK antibodies has been shown to induce apoptosis in different cell types (Hungerford et al., 1996; Otey, 1998). There have also been several reports documenting that FAK associations with both PI3K and p130Cas are important in FAK regulated cell survival (Bellas et al., 2002; Chan et al., 1999). For example, FAK over-expression protects endothelial cells from adenosine (Ado) induced apoptosis, and this protection is dependent on PI3K activity (Bellas et al., 2002).

Finally, FAK has been shown to promote cell survival by affecting p53 transcriptional activity. p53 is a nuclear transcription factor with a pro-apoptotic function, and FAK can both directly bind to p53 and block its transcriptional activity (Golubovskaya et al., 2005). FAK can also bind to Mdm2, a negative regulator of p53, which can lead to proteasomal degradation of p53 (Lim et al., 2008).

1.4.6 FAK and autophagy

One of the proteins that binds to FAK I selected for this study, is an autophagy protein, therefore I will describe this section in more detail.

Autophagy is a cellular degradation pathway that involves sequestration of cytoplasmic materials within autolysosomes in order to maintain cell homeostasis. So far, three types of autophagy have been described: macro-autophagy (autophagy), micro-autophagy and chaperone-mediated autophagy [reviewed in (Boya et al., 2013; Marino et al., 2014)]. One of the first steps of autophagy is the formation of double-membrane vesicles called autophagosomes. Autophagosomes are assembled at the phagophore assembly site (PAS), which is mediated by multiple autophagy-related (Atg) proteins. The nucleation and assembly of phagophore membrane requires

Beclin1/PI3K core complex consisting of PI3K class III (PI3KC3), p150 and Beclin1 [reviewed in (Yang and Klionsky, 2010)]. Beclin1 (also named Atg6) belongs to Atg family of proteins. Beclin1 was shown to restore starvation-induced autophagy in Atg6-disrupted yeast strains, whereas Beclin1 over-expression in human MCF7 breast carcinoma cells lacking detectable levels of endogenous Beclin1 activates autophagy (Liang et al., 1999). Beclin1 is a known interacting partner of PI3KC3. PI3KC3 is a lipid kinase that phosphorylates phosphatidylinositol (PI) to produce phosphatidylinositol 3-phosphate (PI3P). Beclin1/PI3K complex promotes generation of PI3P, which then acts as a platform for Atg and other autophagy proteins [reviewed in (He and Klionsky, 2009)]. p150 is a serine/threonine kinase. It has been shown that p150 recruits PI3KC3 to the membrane, and its intact kinase domain required for PI3KC3 activity (Panaretou et al., 1997). It is considered to be a regulatory subunit of PI3KC3.

More recently, Ambra1 has been identified as a part of the multi-protein core complex (Fimia et al., 2007). It has been demonstrated that Ambra1 plays an important role in embryogenesis, as Ambra1 deficiency in mouse embryos causes neuronal tube defects, which is associated with impaired autophagy. Ambra1 is a positive regulator of autophagy as it binds to Beclin1 and promotes Beclin1 association with PI3KC3 (Fimia et al., 2007).

When autophagosome formation is completed, they are released to the cytoplasm where they fuse to lysosomes. Formation of autolysosomes allows degradation of cargo by lysosomal enzymes. The degraded products are then exported back into the cytosol for reuse [reviewed in (Funderburk et al., 2010)]. For example, amino acids and fatty acids produced by autophagy can be used to synthesise new proteins or are

oxidized by the mitochondria to produce ATP for cell survival. It has been shown that mice lacking Atg5 ($Atg5^{-/-}$) die during neonatal period. These mice exhibit decreased amino acid levels, decreased cardiac ATP production and myocardial damage, demonstrating that recycling function of autophagy is critical to maintain cellular energy homeostasis and cell survival during the neonatal period (Kuma et al., 2004).

Autophagy is a tightly regulated process that plays a role in cell growth, development and homeostasis. Several positive or negative regulators of autophagy have been identified, including UV radiation resistance-associated gene (UVRAG) (Liang et al., 2006), Bax-interacting factor-1 (Bif-1) (Takahashi et al., 2007), and B-cell lymphoma 2 (Bcl-2) (Pattingre et al., 2005). Autophagy was long considered to be a non-selective bulk protein degradation system; however, recent reports clearly indicate that it can also be a highly selective process. Selective autophagy is mediated by specific receptors, which directly bind to a cargo that needs to be degraded [reviewed in (Boya et al., 2013)].

There is evidence that FAK may regulate selective autophagy of active Src in squamous cell carcinoma (SCC) cells (Sandilands et al., 2012a). Recent work in our laboratory has shown that active Src is degraded via autophagy in FAK-/- SCC cells. In SCC cells Src is localised at focal adhesion, however in the absence of FAK autophagy regulators, such as Atg12, Atg7 and LC3, can affect the trafficking of active Src to autophagic puncta (Sandilands et al., 2012a). Localisation of Src is restored to focal adhesions when FAK-WT is re-expressed to FAK-/- SCC cells, or when FAK-/- SCC cells are treated with autophagy inhibitor 3-Methyladenine (3-MA). In addition, transfection of FAK-/- SCC cells with siRNA targeting either Atg5

or Atg12 also restores Src localisation to adhesions. Moreover, it was shown that c-Cbl acts as a cargo receptor when FAK signaling is disrupted, suggesting that FAK regulates selective autophagy of active Src through c-Cbl (Sandilands et al., 2012a)

FAK deletion also results in the selective targeting of Ret for autophagy dependant degradation (Sandilands et al., 2012b). Overall, these experiments suggest that FAK prevents the targeting of active Src or Ret to autophagosomes.

1.4.7 FAK and cancer

FAK is an important mediator of signal transduction by integrins and growth factor receptors, and participates in a wide range of cellular processes including cell proliferation, cell survival, adhesion, angiogenesis and migration. As the development of malignancy is often associated with perturbation in these processes, it is therefore not surprising that FAK activity is altered within cancer cells.

Increases in FAK expression were first observed in high grade and metastatic sarcomas (Weiner, et al., 1994) and since then elevated FAK expression has been reported in many different human cancers based on Western blot and immunohistochemical analysis. High levels of FAK expression have been found in breast (Lark et al., 2005; Pylayeva et al., 2009; Watermann et al., 2005), colon (Owens et al., 1995), prostate (Tremblay et al., 1996), lung (Carelli et al., 2006), gastric (Su et al., 2002), thyroid (Owens et al., 1996), ovarian (Judson et al., 1999) and pre-invasive and invasive oral (Kornberg, 1998) cancers. Moreover, elevated expression of FAK protein, in squamous cell carcinoma (SCC) cells, is due to increased *fak* gene copy number (Agochiya et al., 1999); however, elevated FAK protein expression does not always correlate with copy number increase of FAK gene. For example, FAK expression is increased in tumours containing mutations in

p53. Mutant p53 does not bind to FAK promoter, and therefore does not inhibit its activity compared to wild type p53 (Golubovskaya et al., 2008a).

There is also evidence that not just the over-expression of FAK, but also its phosphorylation and activity is correlated with malignant transformation (Benlimame et al., 2005; Lim et al., 2004). For example, FAK is required for oncogenic transformation and cell invasion mediated by ErbB2/3 receptor signaling, which promotes phosphorylation of FAK at Y397, Y861 and Y925 (Benlimame et al., 2005). In this study, they overexpressed single and paired combinations of ErbB2 and ErbB3 receptors using FAK+/+ and FAK-/- mouse embryonic fibroblasts (MEF), and FAK-/- MEFs in which FAK was reconstituted. They found that FAK deficient cells fail to promote cell transformation and invasion induced by ErbB2/3 receptors; however, the restoration of FAK rescued the ability of ErbB2/3 receptors either to induce anchorage-independent growth on soft agar, or to form lung metastasis after intravenous cell administration. These data suggest the importance of FAK in these processes (Benlimame et al., 2005). It has been shown that increased FAK expression is frequently linked to poor prognosis; however, elevated FAK expression does not predict patient outcome in colon adenocarcinoma patients (Theocharis et al., 2003).

It is noteworthy that mutations in FAK have not been reported in human cancers till recently, when FAK-Del33 mutation was identified in both breast and thyroid cancers (Fang et al., 2014). FAK-Del33 mutation completely deletes exon 33, which is located in the FAT domain of FAK, and leads to enhanced cell motility and migration through modulating FAK/Src signaling pathway. The frequency of fAK-Del33 mutation is ~14.2% in breast carcinomas.

Although FAK overexpression, its phosphorylation and increased activity have been linked with tumour invasiveness and metastasis for many types of cancer, there are some exceptions reported. For example, weak expression of FAK in cervical cancers is correlated with pelvic lymph node metastasis, recurrent disease and poor prognosis (Gabriel et al., 2006). Moreover, FAK dephosphorylation and down regulation of its kinase activity is associated with EGF induced cell invasion and metastasis in human carcinoma cell lines over-expressing the EGFR (Lu et al., 2001). It therefore remains a little unclear how, and when, FAK activity contributes to cancer phenotypes – and there is the suggestion that this may be context-dependent.

1.4.8 *In vivo* study of FAK

The importance of FAK in cancer progression and in angiogenesis has been shown in several animal studies. As genetic deletion of *fak* is embryonic lethal (Ilic et al., 1995) alternative approaches for modulating FAK expression and function has been used including gene targeting, knock-out and inducible expression of genes (Garcia and Mills, 2002).

By using *fak* heterozygous mice (*fak*+/-) with reduced expression of FAK protein, it has been shown that FAK is required for the formation of benign skin tumours called papillomas, in response to DMBA/TPA induced topical carcinogenesis (McLean et al., 2001). However, no malignant conversion was observed in this study due to the fact that FAK protein expression was elevated during papilloma formation, and that both papillomas from *fak*+/- and *fak*+/+ mice displayed equal levels of FAK. Why FAK protein level is elevated during papilloma formation is unclear; however there is a possibility that FAK protein expression is essential for maximal H-*ras* signaling in order to induce key changes within the cell that are required for tumour initiation,

as it has been shown that activation of ERK by activated mutant Ras (Ras/ERK pathway) is still adhesion dependent, and FAK is believed to be upstream of Ras in signaling between integrins and the Ras/ERK pathway (McLean et al., 2001). Later, it was demonstrated that FAK is involved in both papilloma formation and their subsequent malignant conversion to carcinomas (McLean et al., 2004). In this study they used *Cre/lox*P technology to generate mice that are homozygous for floxed *fak* allele and also express 4-hydroxy-tamoxifen (4-OHT) regulated *Cre* recombinase under the control of keratin-14 promoter (K14*Cre*ER^{T2}/FAK$^{flox/flox}$) (McLean et al., 2004). *Cre/lox*P system was originally discovered in bacteriophage P1. *Cre* recombinase is an enzyme that recognises DNA sequence of *lox*P site and mediates integration and excision of DNA into and out of genomes that have *lox*P sites engineered into them. *Cre/lox*P system is functional in mammalian cells and used for generating conditional gene knockout mice models (Garcia and Mills, 2002).

FAK has also been directly implicated in mouse p53-induced mammary tumour development (van Miltenburg et al., 2014). Moreover, by using an inducible mouse model (Pdgfb-iCreER;FAKfl/fl), where FAK is deleted in endothelial cells, it has been shown that FAK is important for directional cell migration in response to VEGF and bFGF, reduced proliferation and increased apoptosis (Tavora et al., 2010). This study suggests that FAK is required for tumour angiogenesis.

Due to its importance in tumour development and progression, several FAK kinase inhibitors have been developed.

1.4.9 FAK inhibitors

Most widely used FAK inhibitors are: TAE226 and PF-562,271, but there are many others from different companies at various stages of pre-clinical or clinical testing.

TAE226 is a small molecular inhibitor that targets the ATP binding site and inhibits FAK phosphorylation at both Y397 and Y861. Large number of studies using TEA226 FAK inhibitor has shown promising results (Golubovskaya et al., 2008b; Halder et al., 2007; Liu et al., 2007; Sakurama et al., 2009; Shi et al., 2007; Wang et al., 2008). However, this drug did not enter clinical trial due to its off-target effects on IGF-R1.

PF-562,271 blocks the ATP binding site of FAK and Pyk2. It has been reported that PF-562,271 inhibits *in vivo* breast cancer cell growth and metastasis through induction of tumour and endothelial cell apoptosis and inhibition of migration (Roberts et al., 2008). Administering of PF-562,271 also prevents TGF-β signaling and metastasis in breast cancer mouse model (Wendt and Schiemann, 2009). Moreover, treatment of mice with PF-562,271 led to significant decrease in tumour invasion and metastasis of pancreatic cancer through inhibiting the migration of cancer cells, cancer-associated fibroblasts and tumour-associated macrophages, suggesting a novel mechanism that PF-562,271 may inhibit cancer metastasis by altering the tumour microenvironment (Stokes et al., 2011). In contrast with TEA226, PF-562,271 has been tested for phase I clinical trials targeting advanced solid tumours (Infante et al., 2012).

Other FAK inhibitors include PF-04554878 and GSK2256098. PF-04554878 is currently in phase I clinical trials for the treatment of advanced non-hematologic

malignancies, while GSK2256098 has completed phase I trials [reviewed in (Dunn et al., 2010)]. Although FAK inhibitors show significant promise for cancer therapy, the nonspecific nature of the ATP binding has raised concerns about potential toxicity.

Most of the FAK inhibitors block its catalytic activity. However, FAK may predominantly act as a scaffolding molecule rather than as a kinase, therefore, disruption of FAK's interaction with protein binding partners could be a good strategy to inhibit some cancer processes. Only a few small molecule inhibitors that target protein-protein interactions (PPIs) have been developed due to the lack of high-throughput screening technologies and the lack of small compounds in compound libraries that could selectively modulate protein complexes. However, several recent studies led to development of appropriate methodologies that should in future lead to identification of potential binding sites on protein-protein interfaces, known as "hot spots" that could be targeted by small compounds (Gonzalez-Ruiz and Gohlke, 2006; Nero et al., 2014). Several small molecule inhibitors targeting FAK scaffolding functions have been developed, such as small molecule compounds C4, INT2-31, M13 and R2 (Golubovskaya et al., 2013a; Golubovskaya et al., 2013b; Kurenova et al., 2013; Ucar et al., 2012). M13 compound targets FAK-Mdm-2 interaction and has been shown to significantly reduce breast and colon tumour growth *in vivo* (Golubovskaya et al., 2013b). Furthermore, R2 compound disrupts the binding of FAK with p53, and in turn increases p53 transcriptional activity and expression of p53-targets, such as p21, Mdm-2 and Bax in colon cancer cells. In addition, R2 blocks tumour growth in HCT116 $p53^{+/+}$ xenografts *in vivo*

(Golubovskaya et al., 2013a). As these compounds do not target ATP binding site, they may provide new opportunities for cancer treatment.

1.4.10 Summary and thesis aims

FAK is a non-receptor protein tyrosine kinase that is involved in many cellular processes including cell proliferation, migration and apoptosis. FAK is elevated in the majority of human cancers. Moreover, strong correlation between FAK expression / phosphorylation and tumour invasive phenotypes has been found. Several FAK inhibitors have been developed, most of which target its catalytic activity. However, FAK kinase activity is not always essential for its signaling function; therefore development of small molecular inhibitors that prevent the association of FAK to its binding partners would block its downstream signaling pathways.

Although there is strong evidence that FAK is an important player in tumour development and progression, the exact mechanism by which FAK can influence the processes involved in tumorigenesis and metastasis requires further studies.

Therefore, in order to gain a better understanding of how FAK may regulate key processes required during tumour formation and progression, the approach adopted was to identify novel protein interactions and study the role of a sub-set of these in tumour biology by impairing them in SCC cancer cells in vitro. SCC cells are a good model for the validation and functional characterisation of novel interactions as SCC cells that are genetically deficient for FAK (FAK-/- SCC cells) and FAK-/- SCC cells re-expressing various FAK mutants enable us to analyse the contribution of individual phosphorylation sites and domains of FAK in the interaction and the downstream signaling events.

To accomplish this, both commercially available human protein arrays and phage display were employed as complimentary approaches in order to identify novel FAK binding partners and assess the role of these in cancer-associated phenotypes.

Many novel interactions were identified and it was decided to focus on two of these, namely Axl and Ambra1, and to study their interactions with FAK and their roles in further detail.

The reasons why these were chosen are as follows: Axl is a protein receptor tyrosine kinase that has previously been linked with tumour progression and metastasis in number of human cancers, and Ambra1 is an autophagy protein that positively regulates early stages of autophagy. Further characterisation of FAK/Ambra1 interaction might help us understand molecular mechanisms of selective autophagy of active Src in SCC cells when FAK is deleted, as reported recently by colleagues in my laboratory.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 General chemicals and reagents

Supplier: ECRC, Edinburgh, UK

Sterile PBS

Sterile PBS/1mM EDTA

Supplier: Invitrogen Life Technologies Ltd, Paisley, UK

DMEM

MEM

MEM vitamins

MEM non essential amino acids

Opti-MEM, reduced serum media

200mM L-glutamine

2.5% trypsin solution

Puromycin

Axl recombinant human protein

Supplier: Sigma Chemical Co, Poole, UK

Foetal bovine serum (FBS)

Sodium pyruvate

Dimethyl sulphoxide (DMSO)

Dithiothreitol (DTT)

Ethylenediaminetetraacetic acid (EDTA)

Glycerol

Lithium chloride

Methanol

Potassium chloride

Sodium dodecyl sulphate (SDS)

Sodium deoxycholate

Sodium chloride (NaCl)

Sodium bicarbonate ($NaHCO_3$)

Sodium azide (NaN_3)

Tris hydrochloride

Ammonium persulphate (APS)

2-mercaptoethanol

Bromophenol blue

Polybrene

Trichloroacetic acid (TCA)

Sulforhodamine B (SRB)

4-hydroxy-tamoxifen

Supplier: Qiagen, Crawley, UK

Polyfect transfection reagent

HiPerFect transfection reagent

Supplier: Merck Biosciences, Sussex, UK

Hygromycin B

Supplier: Melford Laboratories, Suffolk, UK

IPTG

Supplier: Transduction Laboratories, BD Biosciences, Oxford, UK

Fibronectin

2.1.2 Molecular biology techniques

Supplier: Qiagen, Crawley, UK

Miniprep DNA kit

Maxiprep DNA Kit

Gel Purification Kit

PCR Purification Kit

RNA extraction Kit

Superscript First-Strand cDNA synthesis kit

Supplier: Sigma Chemical Co, Poole, UK

Agarose

Ethidium bromide

Coomassie brilliant blue R-250

Supplier: Agilent Technologies, Cheshire, UK

PfuUltra Hotstart PCR Master Mix

Supplier: Quantace Ltd., London, UK

SensiMix™ One-Step kit

Supplier: Ambion Life Technologies, Paisley, UK

TURBO DNA-free™ Kit

Supplier: Invitrogen Life Technologies Ltd, Paisley, UK

PCR/Q-PCR primers

Zero Blunt TOPO PCR cloning kit

One Shot TOP10 chemically competent *E.coli*

One Shot BL21 (DE3) chemically competent *E.coli*

imMedia Growth Medium, agar, kanamycin

imMedia Growth Medium, agar, ampicillin

Supplier: New England Biolabs, Hertfordshire, UK

Not I restriction enzyme

Alkaline phosphatase, Calf Intestinal (CIP)

Hiperladder I molecular weight ladder

Supplier: Roche applied science, West Sussex, UK

Rapid DNA ligation kit

2.1.3 Cell culture plasticware

Supplier: BD Biosciences, Oxford, UK

Falcon tissue culture dishes (60mm, 90mm and 120mm)

Falcon tissue culture plates (6, 12, 24 and 96 well)

Supplier: TCS biologicals, Botolph Clayton, UK

Nunc tissue culture flasks

Nunc cryotubes

Supplier: Corning Life Sciences, Amsterdam, Netherlands

Transwell inserts

2.1.4 siRNA and shRNA

Supplier: Thermo Scientific (Dharmacon), Epsom, UK

SMART pool: siGenome mouse Axl siRNA (M-040941-01-0005)

Set of 4 Upgrade: siGenome mouse Axl siRNA (MU-040941-01-0002)

SMART pool: siGenome mouse Ambra1 siRNA (M-059556-01-0005)

Supplier: Thermo Scientific (Open Biosystems)

Mouse pSM2 retroviral shRNA for Axl (individual clones: RMM1766-96881588, RMM1766-96885903).

2.1.5 Immunofluorescence

Supplier: Olympus UK Ltd, Hertfordshire, UK

Olympus FV1000 Confocal microscope

Supplier: Sigma Chemical Co, Poole, UK

TRITC phalloidin

Formaldehyde

Triton X100

Glycine

EGTA

PIPES

$MgCl_2$

Supplier: Jackson Immunoresearch Laboratories, Luton, UK

Vectashield Microscope Mounting Medium with DAPI

2.1.6 Immunoblot

Supplier: Thermo Scientific Pierce Ltd, Northumbria, UK

Micro BCA Protein Assay Kit

Supplier: Roche Applied Science, West Sussex, UK

cOmplete Protease Inhibitor Cocktail Tablets

PhosSTOP – Phosphatase Inhibitor Cocktail Tablets

Supplier: Invitrogen Life Sciences Ltd, Paisley, UK

NuPage Bis-Tris gels

NuPage MOPS Buffer

NuPage LDS Sample Buffer (4x)

NuPage Electrophoresis Apparatus

Supplier: Protran, Schleicher and Schuell, London, UK

Nitrocellulose membrane

Supplier: Sigma Chemical Co, Poole, UK

Bovine serum albumin (BSA)

Lysozyme

N,N,N',N'-tetramethylethylenediamine (TEMED)

Tween 20

NP-40

Anti-mouse/rabbit IgG (whole molecule) - agarose

Mouse/rabbit IgG agarose

Protein A – sepharose

Supplier: Chemicon International, Harrow, UK

Re-blot Plus Strong Antibody Stripping Solution (10x)

Supplier: GE Healthcare, Little Chalfont, UK

ECL reagent

High molecular weight ^{TM} rainbow markers

Supplier: Whatman, Maidstone, UK

3mm filter paper

Supplier: Thermo Fisher Scientific Loughborough,, UK

X-ray film

Supplier: Jencons, Leighton Buzzard, UK

Wet blotting apparatus

2.1.7 Protein microarray

Supplier: Invitrogen Life Technologies Ltd, Paisley, UK

ProtoArray^{R} Human Protein Microarray PPI Kit

2.1.8 Phage display library

Supplier: New England BioLabs Ltd., Herts, UK

Ph.D.^{TM} – 12 Phage Display Peptide Library Kit

2.1.9 Antibodies

Antibodies for immunoblotting, immunoprecipitation and immunocytochemistry were as follows: anti-FAK, anti-Src 36D10, anti-phospho-Src-Y416, anti-GAPDH, anti-Axl, anti-LC3B, anti-PI3KC3, anti-His-tag, anti-rabbit/mouse (DA1E) IgG XP (^{TM}) isotype control, and anti-mouse and anti-rabbit IgG-HRP conjugated secondary antibodies were from Cell Signaling Technology (Hertfordshire, UK), anti-phospho-FAK-Y397, anti-phospho-FAK-Y576/577, anti- phospho-FAK-Y925, anti-FAK (clone 4.47, used for FAK IPs), anti-Ambra1 antibodies were from Millipore (Bedford, USA). Anti-phospho-FAK-Y861, anti-mouse and anti-rabbit Alexa 488 and 594 were purchased from Invitrogen (Paisley, UK). Anti-β-actin and anti-PIK3R4 (p150) antibodies were from Sigma (Dorset, UK). Anti-RACK1, anti-Beclin1, anti-Paxillin, anti-pY20, and anti-GM130 antibodies were obtained from Transduction Laboratories, BD Biosciences (Oxford, UK). Anti-Axl antibody was from Abcam, Cambridge, UK.

2.1.10 Cell culture solutions

Trypsin

0.25% trypsin in sterile PBS/1mM EDTA

2.1.11 Stock solutions and buffers

Protein extraction

RIPA Buffer

50mM Tris/HCl pH7.4

150mM NaCl

1% Triton X-100

0.5% Sodium deoxycholate

0.1% Sodium dodecyl sulphate (SDS)

NP40 Buffer

150mM NaCl

50mM Tris pH8

0.5% NP40

Western blotting

Acrylamide gel – 7.5%

10ml 30% acrylamide

15ml Tris pH8.8

15ml H2O

400µl 10% SDS

375µl 10% APS

20µl TEMED

Stacker gel

3.2ml 30% acrylamide

2.5 ml Tris pH6.8

14ml H2O

200µl 10% SDS

200µl 10% APS

20µl TEMED

Sample Buffer - 2x

800µl 2-Mercaptoethanol

1.3ml Tris pH6.8

2ml Glycerol

5ml 10% SDS

1.3 ml H2O

Bromophenol Blue to colour

Tank Buffer – 10x

0.05 M Tris

0.05 M glycine

0.1% SDS

Transfer Buffer – 10x

50mM Tris

40mM glycine

0.04% SDS

20% Methanol

Wash Buffer

0.2% Tween 20 in Tris Base solution

Phage display

Stabilizing buffer

0.1M $NaHCO_3$ pH8.6

Blocking buffer

0.1M $NaHCO_3$ pH8.6

5mg/ml BSA

0.02% NaN_3.

Washing buffer

0.1% Tween 20.in Tris Base solution

2.2 Methods

2.2.1 Protein microarray

Protein array experiment was performed using ProtoArray-Human Protein Microarray v5.0 for Protein-Protein Interaction (PPI) kit from Invitrogen. The buffers used in this experiment were supplied in PPI kit. Blocking buffer consisted of 50mM HEPES pH7.5, 25% glycerol, 0.08% triton X-100, 200mM NaCl, 20mM reduced glutathione, 1X synthetic block and 1mM DTT. The washing buffer contained 1X PBS, 0.1% Tween 20, and 1X synthetic block.

In accordance with the manufacturer's instructions the array was placed with the barcode facing up into a well of a chilled 4-chamber incubation tray and blocked with blocking buffer for 1 hour on a shaker set at 50rpm. Purified FAKΔ375 recombinant protein was diluted in washing buffer at a final concentration of ~ 10µg/ml. After blocking, the blocking buffer was removed using a pipette tip and then 120µl of diluted protein was loaded onto the microarray membrane, and the membrane was covered using a Lifterslip. The array was probed for 90 minutes with no shaking at 4^{o}C, then washed five times for 5 minutes with gentle agitation. The array was then probed with anti-FAK (1µg/ml in washing buffer) antibody for 90 minutes followed by five-5 minutes washes, and then probed with anti-Alexa fluor647 (1µg/ml in washing buffer) antibody for 90 minutes. The array was then washed 5 times for 5 minutes in wash buffer and dried by centrifugation at 200xg for 5 minutes. All steps were performed at 4^{o}C except the final step which was carried out at room temperature.

For negative control, the array was probed with the primary and secondary antibodies only.

The arrays were scanned using an Axon GenePix4000B microarray scanner (Molecular Devices) to obtain an array images and the images were saved as 16-bit TIFF files. The scanner settings were as follows: wavelength – 635nm, laser power – 100%, pixel size - 10μm, and PMT gain – 600.

ProtoArray Prospector Imager is a component of the ProtoArray Prospector software package, which allows microarray image processing for data analysis. The raw image from the scanner usually requires some manipulations (rotation, scaling, zoom, colour inversion, or change resolution) before it can be used for signal extraction. After preparing the image of an array, a grid of spots was overlaid onto the image. The grid was loaded from the .GAL (GenePix Array List) file, which describes the location and identity of all spots on the protein array.

Finally, the image files were processed using commercially available ProtoArray Prospector data analysis software (available at www.invitrogen.com/protoarray) to determine signal intensity of the bound protein probe (FAKΔ375) detected by the anti-FAK and anti-Alexa fluor647 antibodies. The basic approach used by the software is to: 1. Calculate the fluorescent signal values taking into account corrections for background and negative control features on the microarray 2. Calculate z-scores for all the corrected intensities and 3. Identify the features that have z-scores greater than cut-off value (3 is the default). These are the protein features scored as positive for interactions.

2.2.2 Phage display

The wells of a 96-well microtiter plate were coated with 150μl of target molecule (FAK-FERM recombinant protein) diluted in 0.1M $NaHCO_3$ pH8.6 at final concentration of ~20μg/ml and incubated overnight at 4^{o}C with gentle agitation. The

coating solution was then removed and the wells were blocked with 150µl/well blocking buffer for 1h at 4°C, followed by washing six times with 200µl/well washing buffer. The washing steps were performed rapidly in order to avoid drying out the plate. Next, the phage library was diluted in TBST (10µl page library + 90µl TBST), then added to the wells and incubated for 1h at room temperature with gentle agitation. Non-binding phage were discarded and the plate washed ten times with washing buffer. After washing off free phage particles, the wells were treated with 100µl of 0.2M Glycine – HCl (pH2.2), 1mg/ml BSA, and incubated for 15 min with gentle agitation at room temperature in order to elute the phage bound to the ligand. The elute then was collected in a micro-centrifuge tube, and neutralized with 15µl of 1M Tris-HCl pH9.1.

To amplify the elute, it was added to the 20ml ER2738 bacterial culture and incubated with vigorous shaking for 4.5h at 37°C. The bacterial cells were then centrifuged for 10min at 12,000 x g at 4°C. 16ml of supernatant was transferred to a fresh tube and 2.7ml (1/6 volume) of 2.5M NaCl in 20% PEG was added to it and kept overnight at 4°C to allow the phage to precipitate. Next day PEG precipitation was centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was removed and the tube was centrifuged again briefly to remove residual supernatant. The phage pellet was suspended in 1ml TBS, transferred to a micro-centrifuge tube and centrifuged at maximum speed for 5min at 4°C. Resulting supernatant was transferred to a fresh tube, 160µl of 2.5M NaCl in 20% PEG added on ice for 1 hour, then centrifuged at 13,000 rpm for 10min at 4°C. The supernatant was discarded and the pellet re-suspended in 200µl of TBS. Amplified page population (10µl of phage

elute) were subjected to further rounds (3 rounds) of binding/amplification cycles to enrich the pool in favour of binding sequences.

For preparation of individual clones for sequencing, serial dilutions (1:10) of phage elution was performed. 10µl of diluted page solution were incubated with 200µl of overnight culture of ER2738 for 5min and then plated on LB agar plates. The phage DNA was then analysed by sequencing. The sequencing primers were supplied by the phage display libraries kit. The selected DNA sequences were translated into amino acid sequences and then compared to a protein database using Basic Local Alignment Search Tool (BLAST) to identify the targets for the FAK-FERM domain.

Phage display experiment was performed once as a screening strategy for subsequent validation by alternative assays.

2.2.3 Generation of recombinant proteins

Generation of Ambra1 mutants

Ambra1 mutants: F1, F2 and F3 (Fimia et al., 2007) were generated by PCR amplification using specific primers. Ambra1 cDNA sequence cloned in pLPCX vector (which was a gift from Prof. Cecconi, Rome) was used as a template. 100ng of DNA and 100ng of each primer was added to PfuUltra Hotstart DNA polymerase in a total volume of 50µl. Samples were preheated at 95^{o}C for 10min before the standard PCR.

The primers were as follows:

Ambra1_F1_F	GCGGCCGCATGAAGGTTGTCCCAGAAAAGA
Ambra1_F1_R	GCGGCCGCCTATTCCTGGGCCTGTTGG
Ambra1_F2_F	GCGGCCGCATGCTCAACAATAACATTGAATCTG

Ambra1_F2_R	GCGGCCGCCTACTCAAAGTCCTCAAATTCCAAG
Ambra1_F3_F	GCGGCCGC ATGTCTGCACCTTCGCTTG
Ambra1_F3_R	GCGGCCGC CTACCTGTTCCGTGGTTCTCC

PCR reactions were ligated into pCR Blunt II-TOPO cloning vector (Invitrogen Life Technologies Ltd, UK) according to manufacturer's instructions and transformed into chemically competent TOP10 bacteria. Transformations were plated on kanamycin (50μg/ml) agar plates to select for positive clones. Isolation of plasmid DNA by mini-prep was followed by NotI restriction enzyme (RE) digestion (1.5 hour, at 37^{o}C). NotI digested F1, F2 and F3 fragments of Ambra1 were gel-purified and ligated into pGex-4T-3 vector at NotI site, placing the insert in frame, by using rapid DNA ligation kit. pGex-4T-3 vectors, expressing Ambra1 mutants, were transformed into chemically competent TOP10 cells and plated on ampicillin (100μg/ml) agar plates to select positive clones. The correct orientation of inserted fragments was confirmed by restriction digest.

pGex vectors expressing FAK mutants, FAK-FERM, FAK-Kinase and FAK-C-terminal were previously generated in the Frame lab.

pGex vector expressing FAKΔ375 mutant was a gift from M. Menza (Beatson Institute).

Expression and purification of recombinant proteins

To express GST-fusion proteins (GST-FAK-FERM/Kinase/C-Terminal, GST-FAKΔ375, GST-Ambra1-F1/F2/F3), pGex vectors were transformed into BL21 (DE3) cells (Invitrogen, UK) in LB medium. Bacterial cultures were grown at 37^{o}C

until OD_{600} reached 0.6, and then GST-fusion proteins were induced by 1mM IPTG at 24°C overnight

The cells from 100ml culture were re-suspended in 50mM Tris, pH8.0, 300mM NaCl, 1mM EDTA, 3mM DTT, 0.1% Triton X100 and lysozyme and lysed by sonication. Insoluble materials were pelleted at 15,000xg for 15 minutes and 200µl of 50% slurry of Glutathione Sepharose 4B beads (GE Healthcare) was added to the clarified supernatants. After 1 hour samples were washed three times in PBS and finally re-suspended in 150ul of PBS. All steps were performed at 4°C unless otherwise indicated.

***Coomassie* staining**

SDS-PAGE gels were rinsed once in dH2O and then 20ml of staining solution (40% methanol, 10% acetic acid, 0.1% Coomassie Blue) added for 2 hours on a shaker at room temperature. Gels were then rinsed twice in dH2O and exposed to de-staining solution overnight (5% Methanol, 7.5% acetic acid).

2.2.4 In vitro binding

FAK-FERM, FAK-Kinase and FAK-C-terminal GST-fusion proteins (5µg) were incubated with 250ng of purified His-tagged Axl ICD (Sigma Chemical Co, UK) in binding buffer (20mM Tris-HCl, pH 7.5, 25mM KCl, 1mM $MgCl_2$, and 50mM NaCl) for 30 min at 4°C with rotation. The samples were then washed three times with binding buffer, boiled in SDS-PAGE sample buffer for 10 minutes, and analysed by SDS-PAGE and immunoblotting using anti-His antibody.

2.2.5 GST pull-down assay

For GST pull-down experiment Squamous Cell Carcinoma cells (SCC) deficient in FAK (FAK-/-), were lysed in RIPA buffer and equal volumes of lysates, containing ~1mg of total proteins, were incubated with GST fusion protein-bound beads (~5µg) for 1 hour at 4^{o}C. Following four washes with ice cold PBS, the beads and precipitated proteins were eluted with 2x SDS-PAGE sample buffer, boiled and loaded onto 10% SDS-PAGE gels for analysis as described in 2.2.13.

2.2.6 Generation of FAK mutant cells

FAK-/- SCC cells expressing FAK mutants were generated by infecting cells with FAK-WT, FAK-Y397F, FAK-Y407F-Y925F or FAK-KD in pWZL retroviral vector. Multiple pools of FAK-WT, FAK-Y397F, FAK-Y407F-Y925F and FAK-KD reconstituted FAK-/- SCC cells were selected, examined for equivalent protein expression and maintained in 0.4mg ml-1 hygromycin B.

2.2.7 Generation of SCC cell lines with stable knockdown of Axl

We obtained two retroviral constracts containing short hairpin RNAs (shRNAs) directed to different coding regions of Axl from Thermo Scientific (Open Biosystems, clones: RMM1766-96881588, RMM1766-96885903). FAK-/- and FAK-WT SCC cells were infected with pSM2 retroviral Axl shRNA vectors. Cells were selected and maintained in puromycin (1.5µg/ml).

2.2.8 Retroviral infection

Phoenix Eco packaging cells were plated on 10cm tissue culture dishes for 24 hours prior to transfection using Lipofectamine 2000 transfection reagent as per manufactures instructions (Invitrogen, UK). Media was removed after 6 hours and replaced with fresh SCC media containing 20% FBS. After 24 hours the viral

supernatant was collected, filtered through 0.45µM membrane and added to SCC cells in the presence of 5µg/ml polybrene. Fresh media was added to the Phoenix Eco cells, a second infection carried out 24 hours later and a third infection 24 hours after that.

2.2.9 Routine cell culture

SCC cells were routinely grown in Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 2mM L-glutamine, NEAA, sodium pyruvate and MEM vitamins in flasks, while Phoenix Eco cells and Ambra1 -/- MEFs were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 2mM L-glutamine. The cells were kept in a dry 37^{o}C, 5% CO_2 incubator. To sub-culture adherent cells the media was removed by aspiration, the monolayer rinsed with PE, then with 10% trypsin/PE solution. Upon detachment the cells were re-suspended with MEM, counted and then transferred into tissue culture flasks or plates.

2.2.10 siRNA

SCC cells were plated overnight on 90mm plates in complete media. 10µl of Axl, Ambra1 or Scrambled siRNA (20µM stock) was mixed with 40µl of HiPerFect transfection reagent in 1ml serum free MEM (SFM) and incubated for 15min at room temperature to allow the formation of transfection complexes. The media from SCC cells was removed and replaced with 4mls of complete media and the siRNA containing solution to give a final volume of 5ml and a final siRNA concentration of 40nM. Media was changed after 24 hours and cells incubated for a further 48 hours at 37^{o}C in CO_2 incubator.

2.2.11 Preparation of protein extracts

Cells were transferred directly from the incubator onto ice, washed twice with ice-cold PBS and harvested in RIPA or NP40 lysis buffer containing freshly added protease and phosphatase inhibitors (Roche Applied Science, UK) for 15 minutes. Cells were scraped and the lysate transferred to a microcentrifuge tube. The lysate was then clarified by centrifugation at 13,000 rpm for 15 minutes at 4^{o}C. The supernatant was collected and protein concentration was determined using micro BCA^{TM} Protein Assay Kit (Pierce Ltd., Northumberland, UK) and the light absorbance then measured with an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany) at a wavelength of 562nm.

2.2.12 Immunoprecipitation

For immunoprecipitation experiments, 1mg of cell lysate was immunoprecipitated with 2µg of primary antibody overnight at 4^{o}C, followed by 60 minutes incubation with 20µl of anti-mouse or anti-rabbit IgG agarose beads. In the case of IgG control, cell lysate was incubated with 20µl of mouse IgG agarose beads. The beads were collected by centrifugation and washed three times with ice-cold lysis buffer, and once with 0.6M Lithium Chloride. Samples were eluted with 20µl of 2x SDS–polyacrylamide gel electrophoresis sample buffer and heated for 10 minutes at 100^{o}C.

2.2.13 Immunoblot analysis

Protein separation was achieved by running 20µg of each lysate and 5µl of pre-stained molecular weight rainbow ^{TM} protein marker (GE Healthcare, Little Chalfont, UK) on either 7.5%, 10%, 12% or 4-12% gradient SDS Page gels. Gels were typically run at 180V for 55min. Following electrophoresis proteins were transferred

to nitrocellulose membranes using wet blotting apparatus with an applied voltage of 100V for 1hr10min. Membranes were then blocked in 5% bovine serum albumin (BSA), re-constituted in TBS plus 0.2% Tween 20 (TBST), for one hour at room temperature with gentle agitation. Primary antibodies (diluted 1 in 1000) in 5% BSA/TBST were added overnight at 4°C, followed by three 10 minutes washes in TBST. Detection was achieved using horseradish peroxidise (HRP)-conjugated secondary antibody for 45 minutes. Membrane was washed again (three times, 15 minutes each wash) and visualized with ECL. When necessary, blots were stripped using the Re-Blot Plus Strong antibody stripping solution (Chemicon International, Harrow, UK) according to the manufacturer's instructions. The blots shown are representative of experiments repeated at least three times except the blot used in figure 4.3(b), which was done twice.

2.2.14 Immunofluorescence

Cells ($5x10^4$) were grown in 12 well plate on glass coverslips, rinsed in 1x TBS, fixed in 3.7% formaldehyde, 100mM Pipes pH6.8, 10mM EGTA, 1mM $MgCl_2$, 0.2% Triton X100 for 10 minutes and washed in TBS with 0.1% Triton X-100. After blocking with 2% BSA/0.1% Triton X-100/TBS for 1 hour at room temperature, cells were incubated with primary antibodies overnight at 4^oC (dilutions shown below). Primary antibody incubation was followed by three washes with TBS containing 0.1% Triton X-100 and subsequent incubation with secondary antibodies conjugated to Alexa 488 or 594 fluorescent dyes (1 in 200) for 45 minutes. Cells were washed 3 times for 5 minutes each and then mounted on glass slides using Immunofluorescence vectasheild mounting medium with DAPI and visualised using an Olympus FV1000 confocal microscope. Images (1024x1024 pixels) were

obtained with a x60 magnification oil-immersion objective. Images shown are representative of the localisation observed in the majority of cells and experiments were repeated at least three times.

Primary antibody dilution for Immunofluorescence

Antibody	Dilution
Anti-FAK	1/200
Anti-phospho SrcY416	1/500
Anti-RACK1	1/100
Anti-LC3	1/100
Anti-Ambra1	1/200
Anti-GM130	1/200
TRITC phalloidin (it's a fluorescent dye)	1/200

2.2.15 Cell polarisation assay

1mg/ml human fibronectin (FN) solution was diluted in sterile PBS to a final concentration of 0.01mg/ml. Coverslips were then coated with FN and incubated at 37^{o}C for at least 1 hour. After washing coverslips twice with sterile PBS, cells were plated on FN at a density of 3 x 10^{6} cells per well on a 12 well dish for 2 hours, in order for cells to form a confluent monolayer. Cells were then wounded using a pipette tip, washed twice in PBS, twice in complete media, and maintained in complete media for 1.5 hours at 37^{o}C. Cells were then fixed as described above and stained for TRITC phalloidin, GM130 and DAPI.

The cell polarisation assay was carried out three times and 150 cells lining the wound edge were counted and analyzed. Cells with the Golgi apparatus orientated towards the wound were scored as positive. Data shown is a mean ± SEM of 3 independent experiments. Statistical tests were performed by using Student's t-test. $P<0.05$ was considered significant and denoted by *.

2.2.16 Cell spreading assay

To investigate cell spreading SCC cells were trypsinized, washed, re-suspended in PBS, and then incubated with rotation at 4^{o}C for 1 hour. The cells were pelleted by centrifugation at 1,000 rpm for 5 minutes, and re-suspended in serum-free medium. Cells ($2x10^5$) were then plated on fibronectin-coated (0.01 mg/ml human FN) coverslips for 30 min and fixed and stained as described above using TRITC phalloidin and anti-RACK1 antibody.

Cells were imaged by confocal microscopy and scored for protrusive structures. Over 100 cells under each condition (for each experiment) were counted in ten independent microscopic fields. Data shown is a mean ± SEM of 3 independent experiments. Statistical tests were performed by using Student's t-test. $P<0.05$ was considered significant and denoted by *.

2.2.17 Invasion assay

Cell invasion was assessed using an *in vitro* inverse invasion assay. 100µl of growth-factor-reduced Matrigel was diluted 1:1 in cold PBS and allowed to set at 37^{o}C for 1 hour in polycarbonate transwells. Cells (1×10^4) were seeded onto the underside of the transwell filter and allowed to adhere for 4h. Transwells were washed and placed in serum-free medium. Normal growth medium containing 10% serum was added above the Matrigel, and cells were allowed to invade for 72 hours. Cells were then

stained with 5μM calcein-AM for 1 hour at room temperature and horizontal z sections through Matrigel were examined at 10μm intervals with an Olympus FV1000 confocal microscope. The amount of dye (positive pixels) was calculated for each z section with ImageJ software and expressed as a percentage of the section that represents the base of the transwell filter. Data shown is a mean ± SEM of 3 independent experiments. Statistical tests were performed by using Student's t-test. $P<0.01$ was considered very significant and denoted by three *.

2.2.18 Sulforhodamine B (SRB) assay

FAK-WT and FAK-WT cells transfected with either scrambled- or Axl siRNA were seeded onto 96-well plates at a cell density of $1x10^3$ cell per well. Cells were fixed at day 0 and day 3 with 25% trichloroacetic (TCA) for 1 hour at 4^oC. The fixed cells were then washed ten times with water and allowed to dry. To stain the cells, 50μl of SRB dye was added to each well. After 30 minutes incubation at room temperature, SRB solution was removed and the plate was washed five times with 1% glacial acetic acid in order to remove unbound dye. The plate was dried and bound SRB dye was solubilised by adding 150μl of 10mM Tris Base buffer (pH10.5) to each well and incubated for 5 minutes at room temperature on a shaker. To determine the optical density of SRB, the plate was read in a 96-well plate reader with the wavelength 540nm. The OD of SRB in each well is directly proportional to the cell number.

SRB assay was performed three times and data are represented as the mean and standard error of the mean (S.E.M.). Statistical tests were performed by using Student's t-test.

2.2.19 Total RNA isolation

RNA was isolated from Keratinocytes, FAK-WT SCC, FAK-/- SCC and MEFs using the Qiagen RNA Extraction Kit (Qiagen, Crawley, UK). To reduce the genomic DNA total RNA was treated with TURBO DNase enzyme (2U) for 20 minutes at 37^{o}C. Afterwards, $1/10^{th}$ volume of reaction mixture of DNase inactivation reagent were added to the samples and further incubated for 5 minutes at room temperature. Samples were then centrifuged at 10,000g for 1.5 minutes and the RNA was transferred to fresh tubes. The total RNA concentration was determined by absorbance measurement (260 and 280 nm).

2.2.20 qRT-PCR analysis

qRT-PCR was performed using the SensiMix One-Step kit., SYBR Green I protocol. Components of master mix used for reaction were 50 x SYBR Green I solutions, Sensi-Mix one step, $MgCl_2$, RNase inhibitor, forward and reverse primers (5µM each) and dH_2O. The primers were as follows:

Axl-Forward: 5' CCCCCGAGGTACTTATGGAT 3'

Axl-Reverse: 5' CAAGTGCTCCCAGCAGTACA 3'

B2M-Forward: 5' CGGCCTGTATGCTATCCAGA

B2M-Reverse: 5' CGGCCTGTATGCTATCCAGA

20µl of PCR master mix and 5µl of RNA template, dH_2O or standards were loaded separately in a duplicate form.

Typical profile times are as follows: Reverse transcription step (one cycle) 42^{o}C 30 minutes; enzyme activation (one cycle) 95^{o}C 10 minutes; amplification (35 cycles), 95^{o}C for 10 seconds (denaturation), 57^{o}C for 20 seconds (annealing); 72^{o}C for 20

seconds (extension). Melting curve 65^{o}C to 99^{o}C, hold every 5 seconds. 72^{o}C for 10 minutes (1 cycle), 15^{o}C for 10 minutes (1 cycle).

Relative fold levels were determined with mB2M used as housekeeping control.

2.2.21 PCR analysis

RNA was isolated from cells and converted to cDNA using Superscript First-Strand cDNA synthesis kit. 2µl of cDNA was added to 18µl of pre-aliquoted master mix (PfuUltra Hotstart PCR Master Mix) containing 100nM of forward and reverse primers. Following amplification, PCR products were separated by agarose gel electrophoresis using a 1.2% agarose gel.

The primers were as follows:

B2M-Forward: 5' GGGAAGCCGAACATACTGAA 3'

B2M-Reverse: 5' TGCTTAACTCTGCAGGCGTAT 3'

Ambra1-Forward: 5' ATGAAAGTTGTCCCAGAGAAGAAT 3'

Ambra1-Reverse: 5' TCATTTTAATGATGTAGATCTTTG 3'

2.2.22 Statistical analysis

Bar charts were created in Excel and represent a mean ± SEM of 3 independent experiments. Statistical tests were performed using Student's t-test, $p<0.05$ was considered significant and denoted by *.

Chapter 3

Protein binding array

3 Protein binding array

3.1 Background

3.1.1 Protein-protein interactions

Proteins are important macromolecules that facilitate most biological processes in a cell, including cell cycle control, cell differentiation, transcription, translation, and post-translational modification. The majority of proteins probably interact with other proteins in order to function properly. Therefore, to fully understand protein function, they can be studied in the context of their interacting partners. Protein-protein interactions can be distinguished based on the lifetime of the complex: a permanent interaction that usually is very stable and a transient interaction (Nooren and Thornton, 2003). Transient interactions play an important role in the regulation of cellular processes and commonly occur between binding domains of signaling proteins, such as SH2 and SH3 (Ozbabacan et al., 2011; Perkins et al., 2010).

Several biochemical, genetic and computational technologies have been developed for the detection of protein-protein interactions. Examples of biochemical interaction technologies are cross-linking, co-immunoprecipitation (co-IP), and the so called pull-down assay, which is similar to co-IP, except that here GST-tagged protein is used instead of antibody. Genetic approaches include yeast two-hybrid, phage display and protein microarrays. Some of these techniques are used for the identification of novel binding partners (Stynen et al., 2012).

In this research protein microarrays and phage display libraries were employed as the major experimental tools, and a brief description of their general properties and functions are given below.

3.1.2 Protein microarrays

For detecting protein-protein interactions, protein arrays offer an *in vitro* alternative method to yeast two-hybrid. Protein microarrays ('protein chips') have been increasingly used for the profiling of protein expression, the determination of protein functions and the identification of molecular interactions between thousands of proteins in a single experiment. In contrast to the yeast two-hybrid system, the *in vitro* nature of protein arrays allows for the control of the protein concentrations, the interaction conditions (pH, temperature, ionic strength) and specific cofactor requirements. A protein microarray consists of a large number of proteins spotted in rows and columns in a very small space with spot sizes less than 250μm (Hall et al., 2007).

Protein chips are prepared by firmly attaching proteins onto the treated surface of microscopy glass slides. Several different slide surfaces are used for protein chips, and these can be divided into three major groups: 1) the first group is two-dimensional (2D) plain glass slides, which bind proteins through the formation of covalent bonds or by electrostatic interactions. 2D glass slides offer strong attachment, however close protein surface contact may affects the three dimensional (3D) structure of proteins; 2) the second group contains 3D gel or membrane coated surfaces, such as poly-L-lysine, agarose and nitrocellulose. These surfaces bind proteins by adsorption. The advantage of 3D surfaces is that proteins preserve their native conformation; 3) the third group includes affinity tag surfaces such as nickel-nitrilotriacetic (Ni-NTA) and avidin slides, and protein attachment to these surfaces is achieved through affinity binding to histidine residues or biotin, respectively. The advantage of affinity tag surfaces is that because proteins are attached through

affinity tag, proteins will be immobilized in uniform orientation where their active sites are exposed to reagent and available to interact with labelled sample (Angenendt, 2005; Bertone and Snyder, 2005; Hall et al., 2007). Regardless of the strategy used, the main aim is to maintain protein in its native conformation so that its binding ability is retained.

It is also very important to block the remaining surface well prior to performing the protein microarray experiment in order to minimise unspecific binding of the target molecules. Labelled target proteins are added to the protein chip which may bind to some of the immobilised proteins. Unbound targets are washed away while bound targets are detected by various methods. The detection of protein binding can be achieved in two ways: 1) directly, by using labelled target molecules (fluorescence labeling, radioisotope labeling), or 2) indirectly in two steps (by using a tagged probe, which can then be detected in a second step using fluorescent secondary antibodies). Fluorescence labeling is the preferred detection method because it is simple, safe and effective (Bertone and Snyder, 2005; Zhu and Snyder, 2003). In addition, fluorescence labels are compatible with laser microarray scanners. The images obtained via microarray scanners are then subsequently analysed with the appropriate software (ProtoArray Prospector Software v5.2).

Over recent years several forms of protein microarrays have been developed: 1) analytical, also known as capture protein arrays, 2) functional protein arrays and 3) reverse-phase protein arrays. 1) Analytical protein arrays display different types of ligands, including antibodies, antigens, aptamers or small molecules on a glass slide. This type of array can be used for monitoring protein expression levels, protein profiling or clinical diagnostics (Phizicky et al., 2003). 2) Functional protein arrays

are used to examine functions of proteins that are immobilized on a glass slide. They display folded and active proteins on the support surface and can be used to investigate protein interactions with other proteins, nucleic acids, lipids and other small molecules (LaBaer and Ramachandran, 2005). In contrast to analytical arrays, functional protein arrays contain full length, functional proteins or protein domains (Merkel et al., 2005). An example of functional protein array used for the detection of protein-protein interaction was presented by Zhu and colleagues. They used protein chips that displayed several thousands of purified yeast proteins to identify calmodulin binding partners (Zhu et al., 2001). These studies identified 39 calmodulin targets in total six of which were known targets (Zhu et al., 2001). 3) In reverse-phase protein arrays, a complex sample, such as a tissue lysate or serum, is immobilized on the glass support surface and targets are then detected with antibodies overlaid onto them. This type of array can be used for the detection of proteins altered as the result of a disease. For example, reverse-phase protein array was used for the identification of novel biomarker proteins of pancreatic cancer (Huang et al., 2014) and chronic myeloid leukaemia (Quintas-Cardama et al., 2012).

The advantages of protein microarrays are:

- It is a powerful technique that enables sensitive, large-scale analysis with economical use of samples and reagents
- Direct target detection
- Lower cost relative to other methods such as a mass spectrometry
- Easy to use

However, despite recent progress, there are number of technical drawbacks:

- In order to spot proteins onto a glass slide they must be purified from cells, and therefore a 'purified' protein may also contain co-purified interacting proteins, which may interfere with protein binding. This can be avoided using *in vitro* expression methods; however, proteins produced this way may not be properly folded.
- As a protein array is an *in vitro* method, interactions that do not normally occur within a cell may take place (false positives) (Chen and Snyder, 2010).
- Lack of post-translational protein modification

3.1.3 Phage display

Phage display was originally developed by George Smith in 1985, when the DNA fragment was inserted into the M13 bacteriophage gene encoding the capsid protein, and the encoded polypeptide was displayed on the surface of phage as a fusion to capsid protein [reviewed in (Pande et al., 2010)]. Since then the technique has evolved and become widely used within biomedical research. Phage display technology represents a system in which bacteriophage display foreign peptides or proteins on their surface, and this is accomplished by incorporating the nucleotide sequence of the protein or peptide of interest into a phage genome as a fusion to the gene encoding the capsid structural protein (Figure 3.1). This fusion ensures that the protein is displayed on the surface of the phage and different sets of genes can be inserted into the genome of multiple phages such that a single phage will only display one protein or peptide on its surface (Willats, 2002). Collections of such phages are described as "libraries". Since the protein or peptide is displayed on the

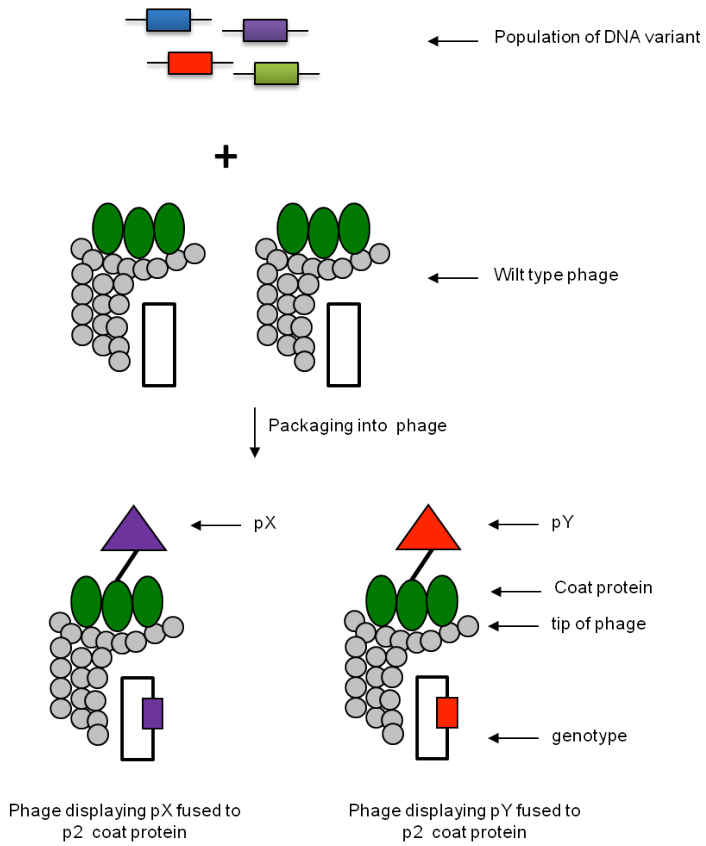


Figure 3.1 A phage display library

A library of DNA sequences encoding peptides or proteins is cloned into a phage genome as a fusion with the coat protein gene. Foreign proteins, pX and pY, are then displayed within the phage coat protein as a fusion protein. [Adapted from (Willats, 2002)].

surface of the phage particle, it is therefore available for interaction with a potential target molecule. Consequently, this method is a particularly convenient tool for understanding protein-protein interactions.

Screening of phage displayed peptide libraries can be achieved through affinity selection process called biopanning (Krumpe and Mori, 2007). Phage clones from the library are incubated with the immobilised target molecule. Unbound phages are washed away and phages bound to the target are recovered by elution. To narrow the diversity of true binding clones, eluted phages are amplified in bacteria, which can be screened again. The screening procedure involves several cycles of selection, until phage pool is enriched in specific binding phage (Krumpe and Mori, 2007).

Phage display peptide libraries have been successfully used in diverse applications, including epitope mapping, vaccine development and identification of novel interacting proteins [reviewed in (Pande et al., 2010)].

There are many advantages to using phage display to study protein interactions:

- The main advantage of phage display is that it provides the possibility of rapidly screening large numbers of proteins or peptides against a potential binding partner
- The technique is relatively simple and inexpensive, can provide results in a matter of weeks and requires no special equipment. It only requires growth of bacteria that is used for amplification of selected phages
- It gives us information about direct interactions
- By linking a selected protein or peptide with its encoding gene, phage display allows us to easily identify DNA sequences of binding proteins or peptides

- The identity of selected peptides or proteins can be simply identified through DNA sequencing.

However, there are also several disadvantages:

- The tightest binding phage may not represent actual biological partners (false positives), meaning that some of the proteins may physically interact, but that such as interaction may not take place physiologically due to the distinct subcellular localisation of these proteins
- False positive results may also appear due to phage binding to other components of the screening system, such as contaminants in the target sample, plastic plates, blocking agents (Vodnik et al., 2011). Some proteins are sticky and they may also generate false positive results
- Phage display screening yields large number of phage clones; therefore it is difficult to assay all of the sequences
- It is an *in vitro* technique and so may not reflect the biological reality
- The lack of post-translational protein modification is an important factor affecting phage display proteins or peptides. It is known that phosphorylation or glycosylation processes play an important role in the functionality of many proteins; therefore its absence could affect the interaction of phage-displayed proteins to appropriate ligands. This is especially true in signal transduction

3.2 Results

As previously described in chapter 1, FAK is crucial for many cellular processes that are disturbed in malignancy, including cell proliferation, adhesion, migration and invasion, thus FAK is important therapeutic target. Several FAK inhibitors have been developed most of which target the FAK kinase function (Golubovskaya, 2014).

Kinase defective FAK retains some of FAK's functions, suggesting that FAK mainly acts as a scaffolding molecule rather than as a kinase (Sieg et al., 2000). In addition, Fan et al. recently demonstrated FAK scaffolding function in breast cancer (Fan et al., 2013). For example, FAK binds to endophilin A2 and mediates its phosphorylation by Src, which leads to increased surface expression of MT1-MMP and promotes invasion of Src-transformed fibroblasts *in vitro*. These authors also showed that when the interaction between FAK and endophilin A2 was disrupted through mutations of amino acids (Pro-878 and Pro-881) within C-terminal proline-rich motif of FAK, mammary tumour growth and metastasis in murine model of human breast cancer was suppressed (Fan et al., 2013). Therefore, disruption of FAK scaffolding function could be a good strategy to anticancer therapy.

The identification and characterisation of novel FAK interactions will aid in uncovering important molecular mechanisms, which may regulate key cellular processes involved in tumour formation and progression, and disruption of these complexes, may improve FAK-targeted cancer therapeutics.

In order to identify novel FAK interactions, the first approach employed was via a protein array.

3.2.1 Identification of FAK Binding Partners Using a Protein Microarray

For this study, the human Protoarray from Invitrogen was chosen, which is composed of over 8000 recombinant proteins of various functional classes spotted in duplicate onto a nitrocellulose coated glass slide. To probe this array, GST-tagged recombinant FAK protein, GST- FAKΔ375, was used, in order to avoid auto-inhibitory interactions between the FAK FERM and kinase domains (figure 4.2).

The GST-FAKΔ375 fusion protein was overlaid onto the protein array and, as a control for non-specific binding partners, a different array was probed with only primary and secondary antibodies. The resulting signals were analysed using the ProtoArray Prospector Software v5.2, which can be set to score positive hits on the protein array. Only duplicate signals of equal intensity, which corresponded to the same protein, were scored as positive. To distinguish false positive signals, the protein array overlaid with GST-FAKΔ375 was compared to the results of the array probed with the primary and secondary antibodies only.

Using this approach, a number of novel candidate binding partners were uncovered in addition to already identified FAK binding partners, such as Src, Fyn and Abl (Table 3.1). Proteins were selected for future study based on reagent availability, protein function and subcellular localisation. One protein chosen for future study was the receptor protein tyrosine kinase, Axl. Similar to FAK, Axl is also involved in the regulation of several cellular processes, such as cell proliferation, cell survival and migration [reviewed in (Paccez et al., 2014)].

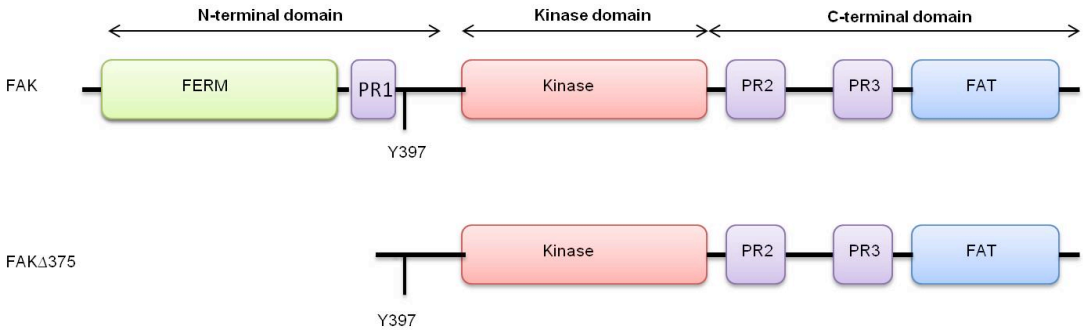


Figure 3.2 Comparison of FAK and FAKΔ375 domain structures

FAK contains three major domains: the FERM, kinase domain and carboxy-terminal domain containing FAT sequence. In FAKΔ375 the FERM domain has been removed in order to avoid auto-inhibitory conformation of FAK.

A number of studies have demonstrated the importance of Axl in cancer. For example, Axl is up-regulated in a vast majority of tumours, including leukaemias, gliomas, melanoma, prostate, pancreatic and breast cancer [reviewed in (Paccez et al., 2014)]. In addition, over-expression of Axl correlates with poor prognosis as well as increased invasiveness (Gjerdrum et al., 2010; Vuoriluoto et al., 2011). Furthermore, a monoclonal antibody that binds to Axl and blocks ligand Gas6 binding to the receptor has been shown to reduce metastasis of MDA-MB-231 breast cancer cells to distant organs (Ye et al., 2010b). These data implied that Axl was a potentially interesting novel FAK binding partner that may be in involved cancer cell behaviours.

No	Symbol	Database ID	Protein Name	Novelty of Interaction
1	PKCɣ	NM_002739	Protein Kinase C gamma	Novel
2	SRPK1	NM_003137	SR protein-specific kinase 1	Novel
3	MAPK2K6	NM_031988	Mitogen-activated protein kinase 2 kinase 6	Novel
4	PKCα	NM_002737	protein kinase C alpha	Novel
5	PIM1	NM_002648	Proto-oncogene serine/threonine protein kinase Pim-1	Novel
6	MAPK2K1	NM_002755	Mitogen-activated protein kinase 2 kinase 1	Novel
7	TSSK2	NM_053006	Testis specific serine/threonine kinase 2	Novel
8	MUSK	NM_005592.1	Muscle, skeletal receptor tyrosine kinase	Novel
9	RS6K	NM_001006932	Ribosomal protein S6 kinase	Novel
10	NEDD9	NM_006403.2	Neural precursor cell expressed developmentally down-regulated protein 9	Novel
11	PKCß1	NM_212535	Protein kinase C beta 1	Novel
12	Axl	NM_001699	Axl receptor tyrosine kinase	Novel
13	ABL1	NM_005157	Abelson murine leukaemia viral oncogene homolog 1	Known
14	Src	NM_005417	Src protein tyrosine kinase	Known
15	FYN	NM_002037	Proto-oncogene tyrosine-protein kinase Fyn	Known
16	STK3	NM_006281	Serine/threonine kinase 3	Novel
17	TGFß1	NM_015927.2	Transforming growth factor beta 1	Novel
18	CK2α	NM_001896.2	Casein kinase 2 alpha	Novel
19	AURKB	NM_004217	Aurora Kinase B	Novel

Table 1 List of the potentially novel FAK interacting proteins identified by Protoarray

3.2.2 Phage display

The second approach used to identify potential FAK binding partners was that of phage display. As described, this technique involves biopanning a random peptide library against a target protein and amplifying the phage-peptides that bind. The process includes eluting and amplifying the phage that binds to a target, plating to yield phage clones for isolating clones DNA, and sequencing of the resultant DNA for characterisation of the peptide. The library used in this experiment was comprised of peptides each containing 12 amino acids.

3.2.2.1 Generation of a bait protein

The FAK FERM domain was used as a bait protein. The FAK sequence encoding amino acid residues 37-413 was amplified by PCR and cloned into the pGex6P3 vector in frame with the GST coding sequence (Serrels et al., 2007). For expression of GST fusion proteins, *E. coli* BL21 was transformed with the vector pGEX6P3-FAK FERM, which carries a *tac* promoter for Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible recombinant protein expression. Following overnight growth and induction of protein expression with IPTG, the bacteria were harvested by centrifugation. Bacterial pellets were then lysed and the clarified supernatant containing the soluble protein extract was incubated with glutathione agarose beads. Bound GST fusion protein was eluted and analysed by SDS-PAGE.

3.2.2.2 Bio-panning and amplification of phage clones

The phage library were then incubated with the bait protein and after a total of three cycles of biopanning and amplification, the eluted phages were cultured with ER2738 and plated in order to select single colonies.

3.2.2.3 Sequencing of DNA inserts

Eleven clones were selected for analysis and the sequence of the DNA inserts was translated to obtain the encoded peptides. After analysing these peptides using the NCBI Basic Local Alignment Search Tool (BLAST) several potential binding partners of the FAK FERM domain were identified (Table 3.2).

Peptides	Potentially Novel FAK Interacting Proteins
LTPSSPASTGNK	MACF1, Ncam1, Liprin, Rptor, Prkcz2, Cttnbp1, c-Abl, Smad5, Laminin_b
YTPSYWGNSLTA	ERBB4, Laminin_a5, CSF2RA, Cntn2, Cntnap3, Inta7
SWGPFTTAPPNN	ProtoCad, Wnk1, Ceacam1, ATG2, LYST
NGPLEIQPSARL	Sorbs3, Myo9a, PIK3CD, Mylk
YIPITTLLTHSH	Stk36, Stk17b
SFPHMTKSHTSN	PdzD2, Otog
YTDSAARSNTVV	Pde4A, Laminin_a2, Cflar, Tpx2, Stk4, Stk3, MVP, Ambra1
SISWQSGHPMSL	Atf7, Otog
VPVHPTPLQPRL	Taf3a, Rnf11, Atf7IP, TBC1, Mll2, Rassf5, Espl1, Arhgap5, Sos1
SILSTMSPHGAT	Neo1, Lyst, Mbnl1
LEASAVYKSKNR	Rin3, Rap1B

Table 2 List of the 12-amino acid sequences identified (left) and the potentially novel FAK interacting proteins (right).

DNA sequences obtained by phage display were translated into amino acids sequences (left) and analysed using NCBI Local Alignment Search Tool. All the proteins in the table are novel potential FAK interacting partners except c-Abl and NCAM1.

3.3 Discussion

A number of studies have highlighted an important role for FAK during tumour development and metastasis; however, the mechanism(s) by which FAK mediates these processes remains to be fully elucidated. The current study was designed to identify novel binding partners of FAK using protein arrays and phage display methodologies.

Initially, we used the human ProtoArray, which is comprised of over 8000 proteins derived from multiple gene families of biological importance, including kinases, cell signalling proteins, metabolic proteins and membrane associated proteins. Arrays such as this have advantages over using conventional proteomics, such as mass spectrometry analysis, as they provide information faster, require less material, and are not prone to contaminants such as keratins. However, they also possess several disadvantages, such as the absence of recombinant protein post-translational modifications and a lack of information as to under what conditions any potential interactions may occur, in addition to fact that only direct interactions can be detected and not complexes. Therefore a number of other approaches, including mass spectrometry, would be optimal.

Using the ProtoArray we identified both known FAK binding proteins, such as Fyn, Src, PKC and Abl, in addition to a number of unknown binding partners with a variety of functions. These proteins included receptor and non-receptor protein kinases, for example 1) receptor protein tyrosine kinase Axl regulates many cellular processes (cell survival, cell proliferation and migration) (Korshunov, 2012); 2) casein kinase II subunit alpha (CK2α) is a serine/threonine protein kinase and

regulates numerous cellular processes (cell cycle progression, apoptosis and transcription) (Duncan and Litchfield, 2008); 3) aurora kinase B (AURKB) is part of the chromosomal passenger complex (CPC) and acts as a key regulator of mitosis (Xu et al., 2012); 4) serine/threonine-protein kinase 3 (STK3) has been shown to play a critical role in tumour suppression by restricting proliferation and promoting apoptosis, and 5) ribosomal protein S6 kinase (RS6K) acts downstream of mTOR signaling and promotes cell proliferation, cell growth and cell cycle progression; etc (Magnuson et al., 2012). From the list of potential FAK binding partners, the receptor protein tyrosine kinase Axl was selected and further studies on the interaction of FAK/Axl are described in Chapter 4.

As mentioned above, there are disadvantages when using protein arrays to look for novel protein-protein interactions. Therefore phage display was also employed as a second approach using FAK-FERM domain this time, although similar to every screening approach false positives may be detected. These can occur for a variety of reasons, for example an irrelevant interaction between a phage displayed peptide and the bait. Furthermore, each phage only displays a 12 amino acid fragment of a protein, which could result in conformational alterations and thereby influence binding ability. Thus, it is essential that interactions identified in a phage display screen are confirmed by at least one alternative assay.

Using phage display, several previously reported FAK binding proteins were detected, such as cAbl and NCAM1. Proteins that are involved in various cellular processes that have not been previously described in association with FAK were also identified. For example, 1) microtubule-actin cross-linking factor 1 (MACF1) belongs to the plakin family of cytoskeletal linker proteins and has recently been

shown to be involved in the Wnt signaling pathway (Chen et al., 2006); 2) Smad5 is a component of the intracellular signaling of transforming growth factor-beta (TGFβ) family members, and TGFβ signaling has been shown to promote tumour development and progression (Lebrun, 2012); and 3) Sorbs3 (which is also known as Vinexin) is a cytoskeleton protein localised at focal adhesions and plays a role in cell spreading, migration and anchorage-independent growth (Mizutani et al., 2007).

Proteins involved in autophagy were also identified, such as, autophagy-related protein 2 (Atg2) and activating molecule in Beclin1-regulated autophagy protein 1 (Ambra1). The Atg2 protein is required for vesicle nucleation/formation during autophagy and is necessary for the localisation of Atg18 to the pre-autophagosomal (PAS) structure and the binding of Atg18 to Atg9 (Feng et al., 2014). Ambra1, which was also identified, promotes the interaction between Beclin1 and VPS34, therefore mediating autophagosomes nucleation (Fimia et al., 2007).

How FAK regulates selective autophagy of active Src is not currently clear and so this study examined Ambra1 as a potential FAK binding partners that are involved in autophagy. We focused on Ambra1 protein because it had been reported that Ambra1 plays a key role in autophagy-dependent protein turnover, and that it's a positive regulator of early stages of the autophagy pathway (Fimia et al., 2007). This work is described in Chapter 5.

Protein microarrays and phage display were used to identify novel FAK binding partners. We identified several proteins by using each tool. However we did not detect any protein that was present in both experimental results. This could be due to the different FAK domains used as bait (GST-FAK-FERM and GST-FAKΔ375) for

these experiments. Moreover, we were not able to sequence all the colonies produced by phage display. Even though both techniques provide quick and simple strategies for examination of protein-protein interactions on a large scale, both protein microarray and phage display lack posttranslational protein modifications, which are often important for many protein functions. Therefore, using mass spectrometry for identification of novel protein-protein interactions would be ideal.

Chapter 4

The interaction between FAK and Axl

4 The interaction between FAK and Axl

4.1 Results

4.1.1 Generation of FAK deficient SCC cells

In this study I used SCC cells that had been generated from chemically induced SCC in mice. These animals expressed in their skin a floxed form of *fak* under the control of *Cre* recombinase. The *lox*P sites were introduced into *fak* gene at the position containing amino acids 413-433, which corresponds to the exon encoding part of the kinase domain (McLean et al., 2004). *Cre* recombinase was regulated by the skin specific keratin-14 promoter (K14*Cre*ER^{T2}/FAK$^{flox/flox}$), which is highly active in dividing cells of the epidermis from day E14.5 (Vasioukhin et al., 1999). *Cre* was fused to the mutated hormone-binding domain of the human estrogen receptor (ER), in which the amino acid corresponding to Glycine 521 (G521) was mutated to an Arginine (G521R). G521R mutant does not bind to 17β-estradiol, but it does bind to synthetic ligand tamoxifen or 4-hydroxy tamoxifen (4OHT) (Feil et al., 1996). It has been shown that the fusion protein *Cre*-ER^{T} is functional tamoxifen dependent recombinase in transgenic mice (Feil et al., 1996). A schematic representation of K14*Cre*ER^{T2}/FAK$^{flox/flox}$ system is shown in Figure 4.1.

The K14*Cre*ER^{T2}/FAK$^{flox/flox}$ mice were then subjected to a two-stage chemical carcinogenesis. Single treatment with 7,12-dimethylbenzanthracene (DMBA) results in an activating mutation of the *Harvey Ras (H-Ras)* gene, and subsequent repeated treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) induce the formation of benign papillomas, which then progress into SCC (McLean et al., 2004).

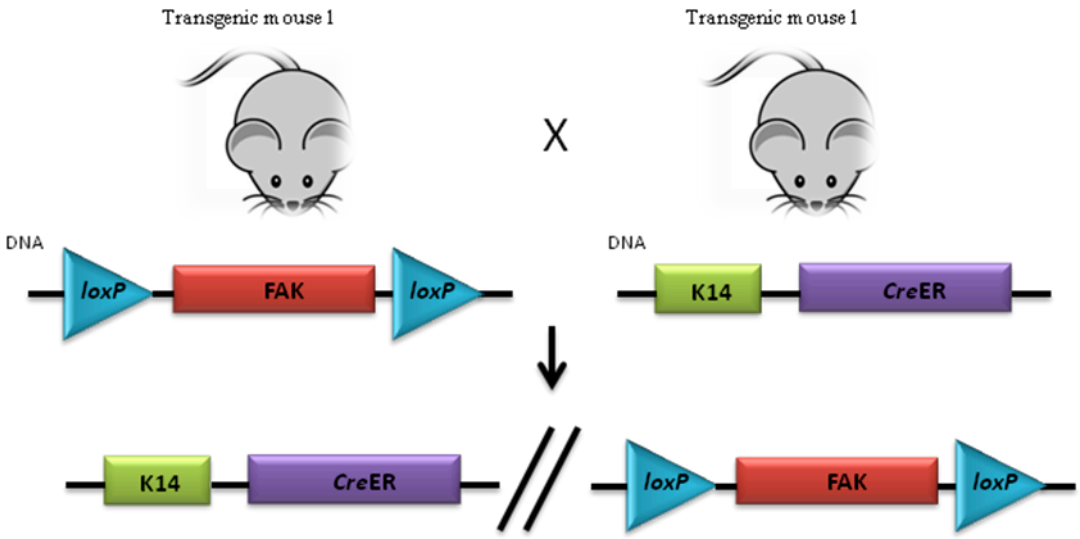


Figure 4.1 Schematic diagram of the K14*Cre*ER/FAK$^{flox/flox}$ system used

The FAK deficient (FAK-/-) cells were generated by treatment of SCC cells with 4OHT. Cells were then sub-cloned and selected by analysing clones for FAK expression/deletion (Serrels et al., 2012). To confirm loss of the FAK protein, FAK-/- SCC cells were lysed and analysed by immunoblotting using anti-FAK antibody. The blot showed that FAK was completely deleted in FAK-/- cells (Figure 4.2a). The blot was also probed with an anti-actin antibody to demonstrate that equal amount of lysates were loaded onto the gel. Immunoblot analysis of FAK-/- cell lysates using an antibody to the carboxyl terminus of FAK also revealed that FAK-/- cells do not express either FRNK or other transcript variants of FAK.

The FAK status was also analysed by immunofluorescence (IF), where FAK+/+ and FAK-/- SCC cells were fixed and stained using a rabbit polyclonal anti-FAK antibody. It was found that FAK was localised at focal adhesions in FAK+/+ cells while it was not visible in FAK-/- cells (Figure 4.2b).

FAK-/- cells were used to generate SCC FAK-WT cells by re-expressing the wild type FAK (FAK-WT) protein. The re-introduction of FAK-WT was achieved through the generation of stable cell lines using the hygromycin B-resistant pWZL retroviral vector (Sandilands et al., 2012a). Following retroviral infection of FAK-/- cells, FAK-WT expressing cells were identified by selection using hygromycin B. The stably expressing FAK-WT protein level was detected by immunoblot analysis using an anti-FAK antibody, and an anti-actin antibody was again used as a loading control (Figure 4.2c). We think that SCC cells are a good model to study FAK/Axl interaction as FAK-/- SCC cells allow generation of different FAK mutant-expressing SCC cells that will let us analyse the contribution of FAK

phosphorylation or its kinase activity in the regulation of this interaction and the role of FAK/Axl interaction in SCC cell biology.

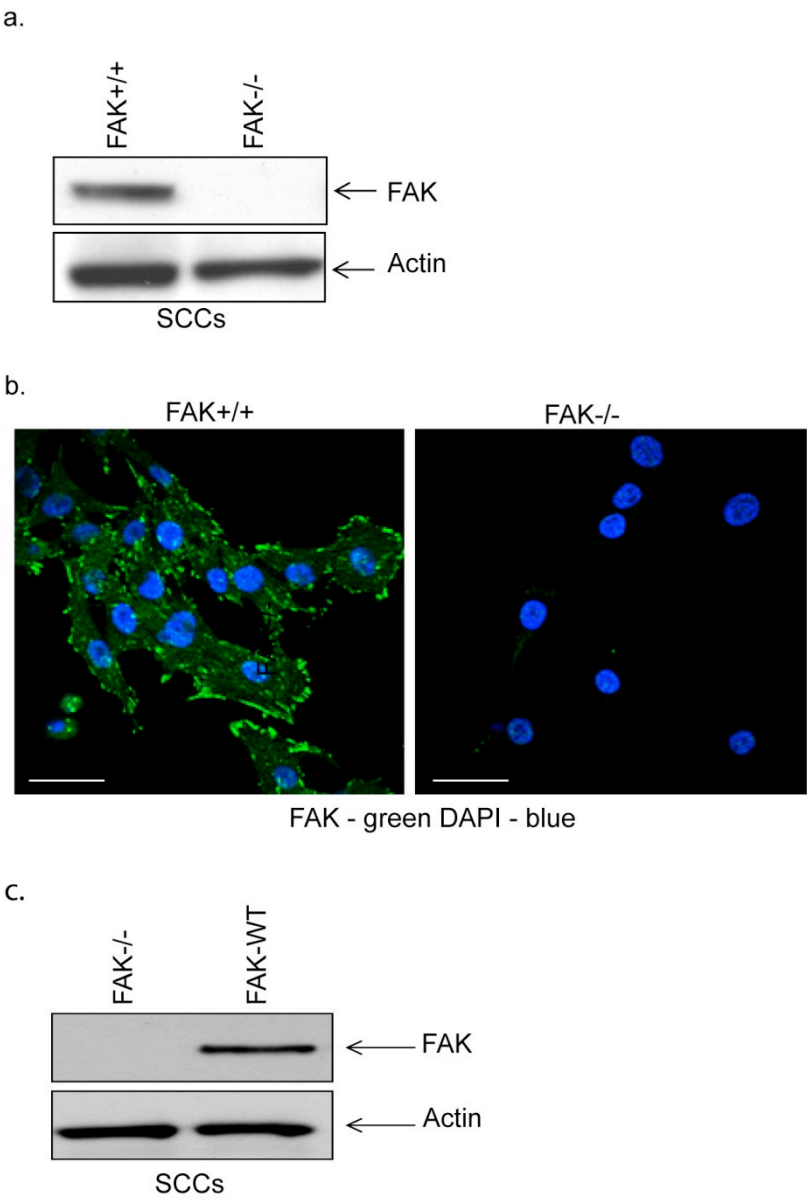


Figure 4.2 Characterisation of SCC cells

(a) Protein extracts from SCC FAK+/+ and FAK-/- cells were separated by SDS-PAGE, transferred to nitrocellulose, blotted and probed with either anti-FAK (upper panel) or anti-actin (lower panel). (b) FAK+/+ and FAK-/- cells were fixed and stained with anti-FAK (green) and DAPI (blue) prior to visualisation by confocal microscopy. (c) Protein extracts from FAK-/- and FAK-/- cells stably re-expressing FAK-WT protein were immunoblotted with anti-FAK antibody. Actin loading is also shown.

4.1.2 Axl is up-regulated at both the RNA and protein levels in SCC cells

Over-expression of Axl has been reported in many human primary cancers, including squamous cell skin cancers (Cichon et al., 2014; Green et al., 2006). To identify whether Axl gene expression was altered in mouse SCC cells compared to normal skin keratinocytes, quantitative (q-) RT PCR was performed. Gene expression was normalised against the housekeeping gene, mouse beta-2 microglobulin (mB2M). The q-RT PCR data demonstrated that Axl mRNA expression was significantly upregulated (>10 fold) during in SCC cells derived from DMBA/TPA model when compared to keratinocytes (Figure 4.3a). This finding is in agreement with previous studies (Cichon et al., 2014; Green et al., 2006) and immunoblot analysis was performed to confirm this finding at the protein level (Figure 4.3b).

The full-length human Axl protein contains 894 amino acids (879 aa in mouse) and has a molecular weight of 104 kDa (97 kDa in mouse). However, it has been shown that the extracellular domain of Axl contains six N-linked glycosylation sites, and two other post-translationally modified forms (100 and 130 kDa) have also been identified, representing partial and complete glycosylation (Li et al., 2012). In SCC cells Axl appears as multiple species, ranging from ~55 kDa to 130 kDa (Figure 4.3b), from which 55 kDa and 130 kDa species are most prominently expressed. Axl antibody specificity was confirmed by Axl siRNA transfection of FAK-WT and FAK-/- SCC cells (Figure 4.3d). The results indicated that Axl expression was upregulated at the protein level, representative example of which is shown in Figure 4.3c. Taking all of these findings into consideration, SCC cells are an interesting model for the validation and functional characterisation of the FAK/Axl interaction.

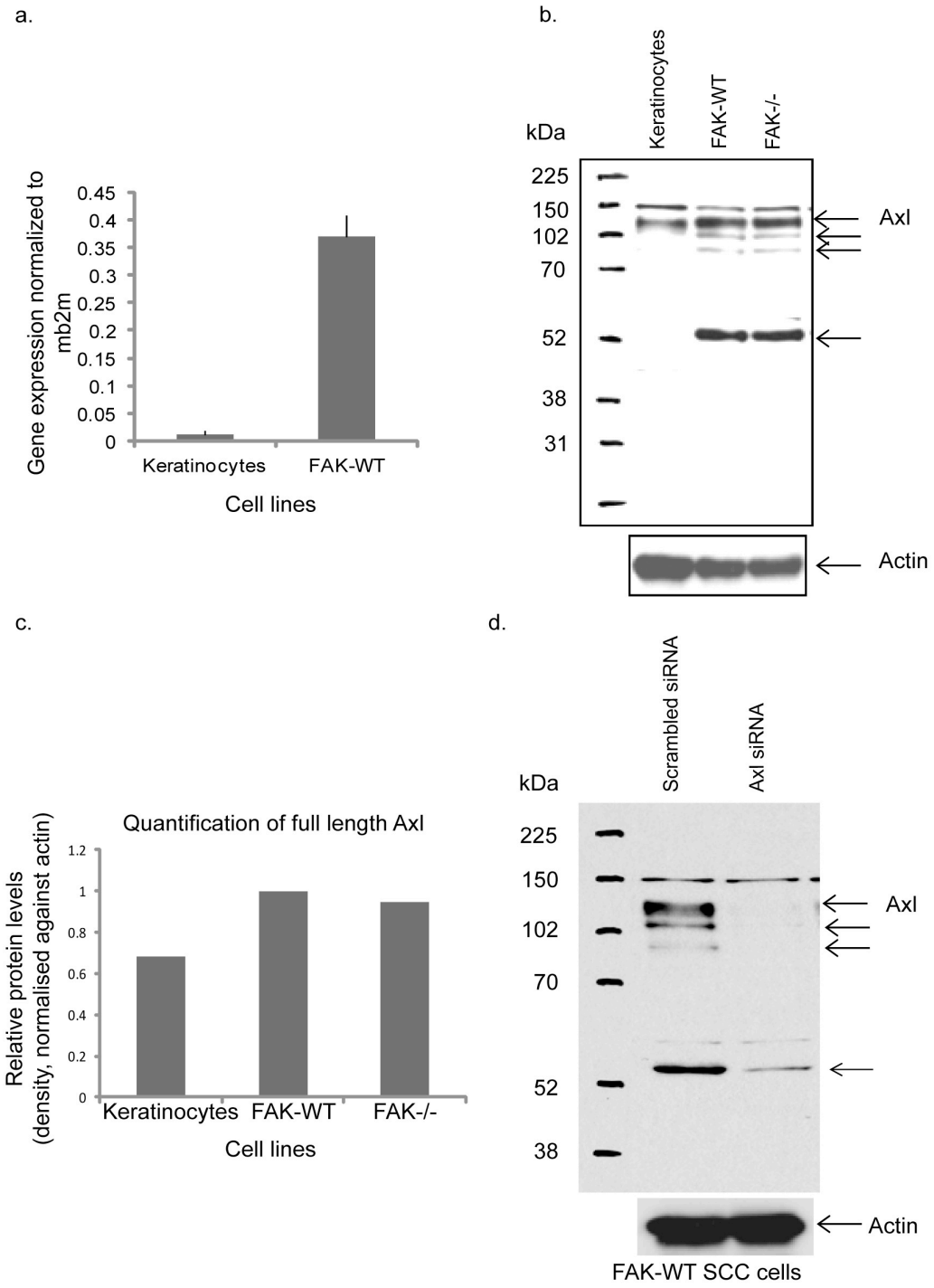


Figure 4.3 Axl is up-regulated at both RNA and protein levels in SCC cells

Quantitative RT-PCR of Axl gene expression in keratinocytes and FAK-WT SCC cells (b) Immunoblot analysis of Axl protein expression in keratinocytes versus FAK-/- and FAK-WT SCC cells. Actin blot is also included (c) Quantifications of full length Axl protein in keratinocytes versus FAK-/- and FAK-WT SCC cells (number of replicates = 2). (d) FAK-WT SCC cells were transfected with Axl siRNA. 72 hours after transfection cells were lysed and subjected to immunoblot analysis. The resulting protein blot was probed with anti-Axl and anti-actin antibodies. Scrambled siRNA were also included as a control.

4.1.3 GST pull-down

To test the interaction between FAK and Axl and determine the regions of binding in FAK, glutathione S-transferase (GST) pull-down experiments were performed using a GST-tagged fragment of each individual domain of FAK and GST-alone.

GST-FAK fusion proteins and GST-alone (Figure 4.4a) were expressed in *E. coli*, and then purified and incubated with the FAK-WT cell lysates. After pull down of GST-fusion proteins and GST as a control, the samples were analysed by immunoblotting using an anti-Axl antibody (Figure 4.4b). The Axl protein was pulled down with some of the GST-fusion proteins to a greater or lesser extent, but not by GST alone, thus supporting the interaction of these two proteins that had been identified by protein microarray analysis. The results also demonstrated that there may be multiple regions within FAK that can bind to Axl, but it is likely that the kinase domain is a major component mediating FAK/Axl interaction.

4.1.4 *In vitro* binding assay

To determine whether FAK/Axl interaction is a direct physical interaction between these two proteins, *in vitro* binding assays were conducted using purified GST-fusion fragments of individual FAK domains and the His-tagged Axl intracellular domain (His-Axl ICD).

GST-fusion FAK domains conjugated to glutathione agarose beads were incubated with the His-tagged Axl intracellular domain containing amino acids – 473-894, and bound proteins were immunoblotted using anti-His antibody. Glutathione agarose beads conjugated to GST alone were also used as a control to confirm that the binding of the His-tagged Axl ICD was due to its binding with FAK, but not to GST.

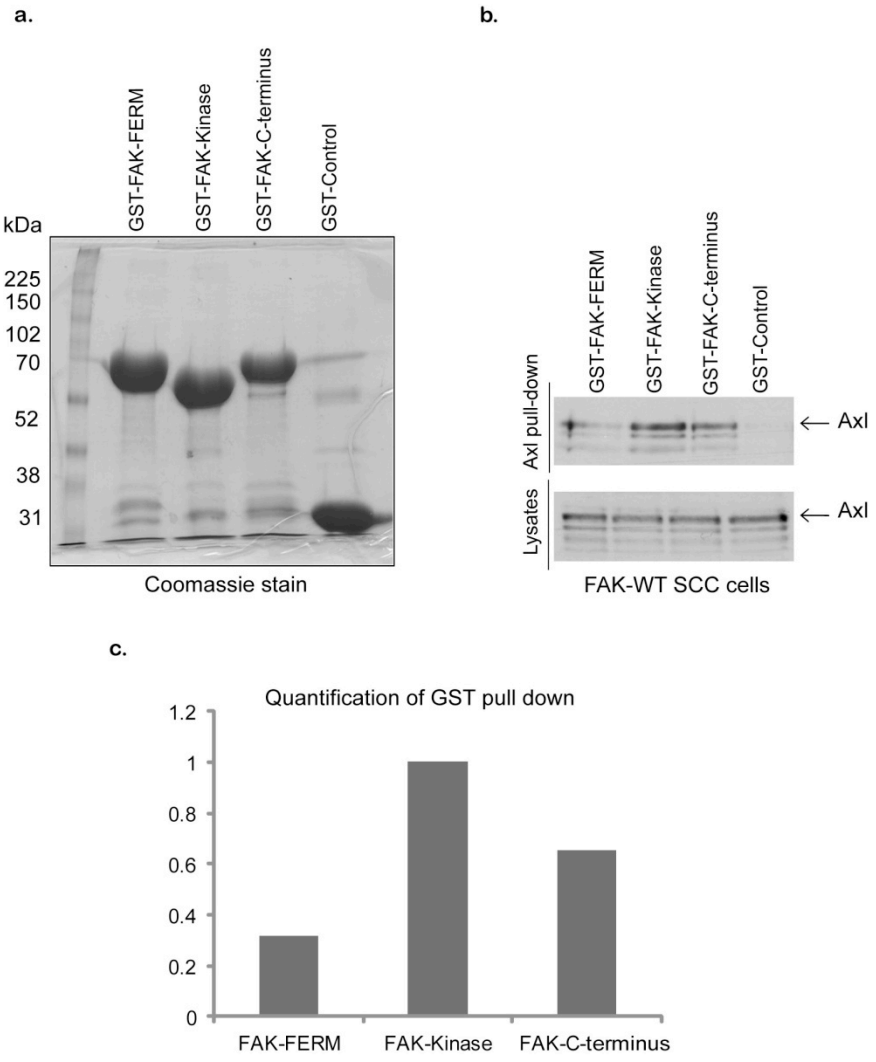


Figure 4.4 Axl pull-down

a) Fusion proteins produced in bacteria were separated by SDS-PAGE and stained with Coomassie Blue. b) FAK-WT SCC cell lysates were incubated with purified GST-FAK-FERM, GST-FAK-Kinase, GST-FAK-C-terminus and GST- itself as a control. Bound proteins were detected by immunoblotting using anti-Axl antibody c) Representative example of quantitative analysis of GST pull-down is also shown.

The results demonstrated that the His-tagged Axl ICD did interact with GST-FAK-kinase; there was also detectable interactions between other GST-FAK domains and His-Axl ICD but not with the GST negative control protein (Figure 4.5a), hence supporting the data identified by GST-pull down experiment, that there may be multiple binding sites within FAK that binds to Axl, but the kinase domain binds more robustly (Figure 4.5b).

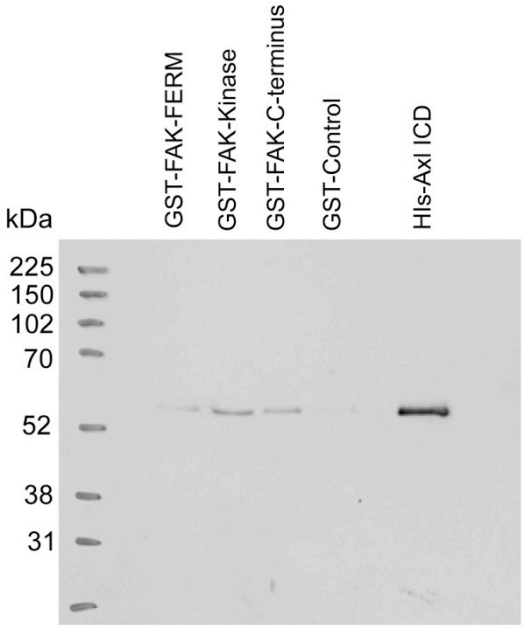
4.1.5 Immunoprecipitation analysis validated the specific binding of Axl to FAK

To test whether FAK and Axl are associated in complex within SCC cells described here, co-immunoprecipitation (co-IP) studies using FAK-WT cells were performed. Co-IP of cell lysates with an anti-Axl antibody was followed by immunoblotting with either anti-Axl or anti-FAK antibodies as probes, which confirmed that FAK does bind to Axl in mammalian cells. In contrast, co-IP with a non-immune IgG control did not precipitate FAK (Figure 4.6).

4.1.6 Regulation of the FAK/Axl interaction

Next, we examined whether the interaction between FAK and Axl proteins was regulated by FAK-Y397 phosphorylation, Src dependent phosphorylation of FAK or by FAK's kinase activity. This was achieved using FAK-/- cells expressing FAK-WT, FAK-Y397F (an auto phosphorylation site mutant that has an impaired response to integrin signaling), FAK-Y4F-Y9F (which has all of the Src-dependent phosphorylation sites mutated to phenylalanine, and so cannot be phosphorylated by Src), and FAK-KD (as a kinase-defective version of FAK due to the mutation of lysine (K) 454 to arginine (R) in the ATP loop (as used before in Sandilands et al., 2012; Serrels et al., 2010).

a.



b.

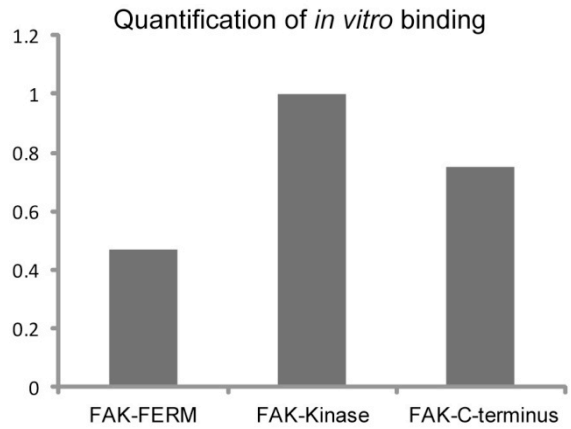


Figure 4.5 In vitro binding

a) GST-FAK-FERM, GST-FAK-Kinase, GST-FAK-C-terminus and GST-itself fusion proteins were incubated with His-Axl ICD. Bound proteins were immunoblotted using anti-His antibody. b) Representative example of quantitative analysis of in-vitro binding is also shown.

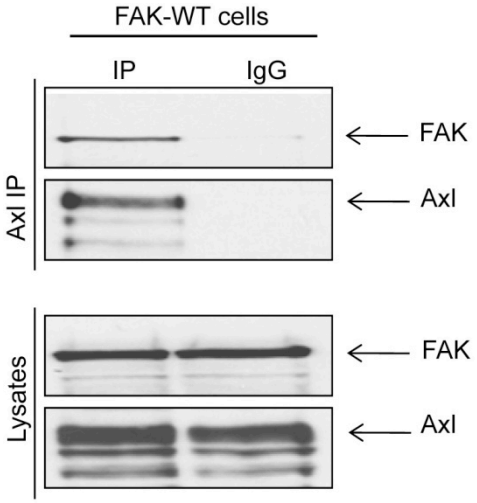


Figure 4.6 Interaction between FAK and Axl proteins

Cell extracts from FAK-/- SCCs re-expressing FAK-WT were immunoprecipitated with anti-Axl antibody. The immunocomplexes were then analysed by immunoblotting with anti-FAK and anti-Axl antibodies. Total cell lysates were also directly probed with the same antibodies. IgG control is also included.

To generate FAK mutant cell lines, FAK-/- cells were infected with pWZL retroviral vectors encoding FAK-Y397F, FAK-Y4F-Y9F and FAK-KD tyrosine kinase mutant cDNAs (Sandilands et al., 2012a). Infected cells were selected using hygromycin B to provide a pooled clone of each. Stable expression of the FAK-WT or mutant proteins was confirmed by immunoblotting (Figure 4.7a, lower panel). SCC cell lysates were also used in immunoblotting experiments, where they were probed using phospho-specific antibodies to FAK-Y397, Y576/577 or Y925. FAK-WT was tyrosine phosphorylated on all residues, and phosphorylation of Y397 in cells expressing FAK-Y397F was not detected since this residue had been mutated in this protein (Figure 4.7a, upper panel). SCC cells expressing the FAK-Y397F mutant also demonstrated reduced phosphorylation of FAK-Y576/577 and FAK-Y925 when compared to FAK-WT. This was expected, since Y576/577 and Y925 are Src dependent phosphorylation sites (Calalb et al., 1995; Schlaepfer and Hunter, 1996), and the binding of Src to FAK protein requires phosphorylation of Y397 (Schaller et al., 1994, Xing et al., 1994). Src binding to phosphorylated Y397 leads to phosphorylation of the remaining tyrosine residues within FAK (Calalb et al., 1995). The Y397 residue was phosphorylated in FAK-Y4F-Y9F mutant protein; however, there was no detectable phosphorylation of Y576/577 and Y925 as expected. The kinase defective FAK-KD mutants showed reduced phosphorylation of all tyrosine residues compared to FAK-WT.

To determine whether the FAK/Axl interaction was influenced in SCC cells expressing FAK mutants, co-IP experiments were performed. Cell lysates from FAK-WT, FAK-Y397F, FAK-Y4F-Y9F and FAK-KD expressing SCC cells IP-ed with an anti-Axl antibody and then immunoblotted with anti-FAK and anti-Axl.

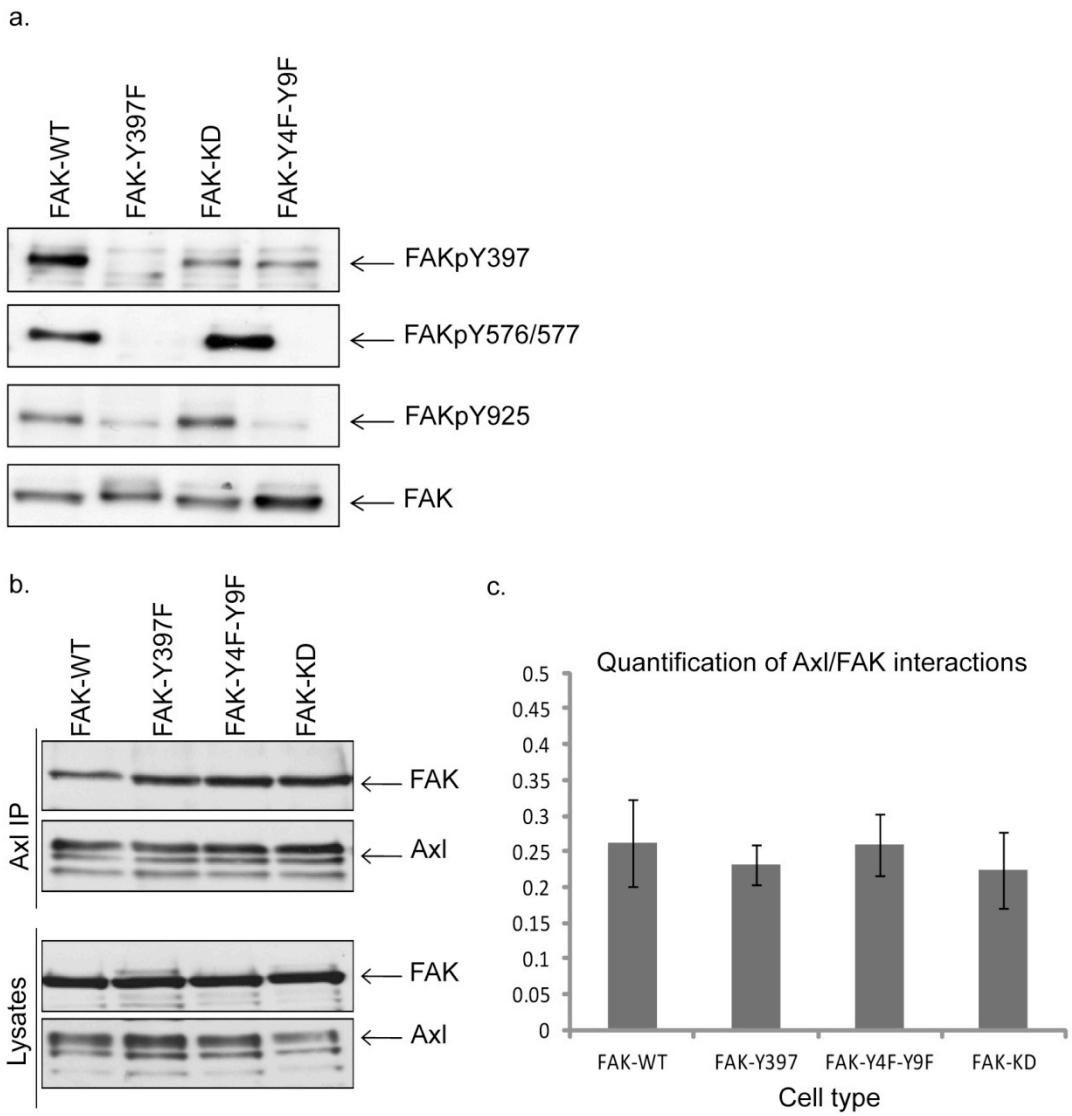


Figure 4.7 Regulation of FAK/Axl interactions

a) Lysates from FAK-/- SCC cells stably re-expressing FAK-WT, FAK-Y397F, FAK-Y4F-Y9F and FAK-KD were immunoblotted with anti-FAK, anti-FAKY397, anti-FAKY925 and anti-FAKY5576/577 antibodies. b) Cell extracts from FAK-/- SCCs re-expressing FAK-WT, FAK-Y397F, FAK-Y4F-Y9F and FAK-KD were IP with anti-Axl antibody. The immunecomplexes were then analysed by immunoblotting with anti-FAK and anti-Axl antibodies. Total cell lysates were also directly probed with the same antibodies. (c) Quantification of Axl/FAK interactions. Columns and error bars represent the mean ± SEM (n=3). Statistical significance was calculated using a student t-test.

The results indicated that there was no significant difference in FAK/Axl binding in the different FAK mutant cell lines compared to FAK-WT (Figure 4.7b). Therefore, we concluded that this interaction was not regulated by FAK-Y397 phosphorylation, Src dependent phosphorylation of FAK, or FAK's kinase activity. Hence, it appears that the FAK/Axl interaction is predominantly a scaffolding function of FAK and seems to be un-regulated by phosphorylation, at least not by any of the major phosphorylation events characterised for FAK so far.

4.1.7 Axl has no effect on the phosphorylation of FAK

Since Axl is a receptor tyrosine kinase, we next investigated whether the physical association of Axl with FAK could be an important trigger for FAK-phosphorylation. To address this question, Axl siRNA was used to robustly reduce expression of Axl protein in FAK-WT SCC cells (Figure 4.8a) and cell lysates were analysed by immunoblotting using phospho-specific antibodies for FAK in order to obtain a FAK phosphorylation profile. Scrambled siRNA was also used as a control (Figure 4.8b). The results show that the levels of Y397, Y576/Y577, Y861 and Y925 phosphorylation between scrambled and Axl-siRNA transfected cells remained unchanged, suggesting that Axl has no effect on FAK phosphorylation.

4.1.8 Downregulation of Axl has no effect on SCC cell proliferation

To examine whether Axl plays a general role in the regulation of SCC cell proliferation, an SRB assay was performed using Axl knockdown FAK-WT SCC cell lines that were generated using Axl specific siRNA. To ensure that knockdown of Axl expression was achieved successfully, the time-course of altered Axl expression following siRNA treatment was analysed.

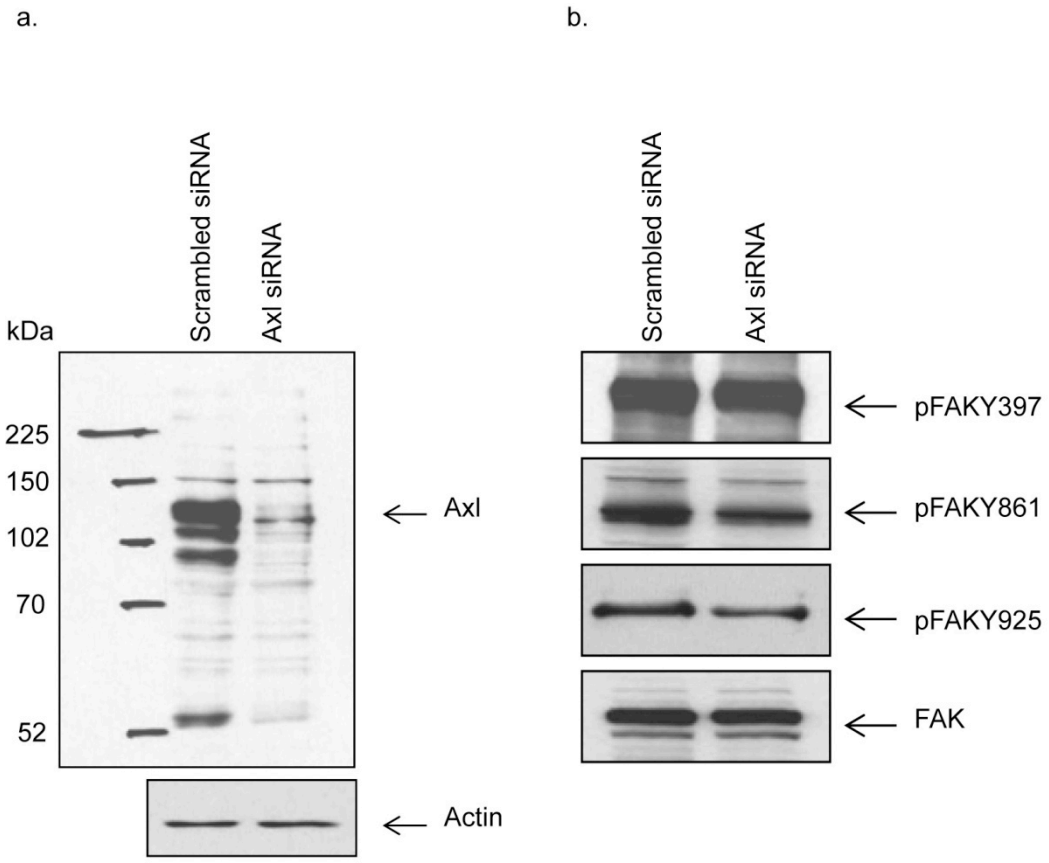


Figure 4.8 Reducing of Axl expression has no effect on FAK phosphorylation

(a) Analysis of siRNA-induced alterations of Axl protein expression by immunoblotting. Protein extracts from FAK-WT cells transfected either Scrambled or Axl siRNA were immunoblotted with anti-Axl antibody. Immunoblotting of actin is also shown (lower panel). (b) FAK-WT SCC cells transfected with either scrambled or Axl siRNA were lysed and immunoblotting carried out using anti-pFAKY397, anti-pFAKY861, anti-pFAKY925 and anti-FAK antibodies.

The protein extracts from FAK-WT cells transfected with either scrambled or Axl siRNA were subjected to SDS phage and immunoblotted with anti-Axl and anti-actin antibodies as probes. The results in Figure 4.9a show decrease in Axl expression 24 hours after siRNA treatment and this remained constant throughout the 72 hour time course (Figure 4.9a).

FAK-WT cells were transfected with either scrambled or Axl siRNA. After 24 hours, cells were trypsinized and seeded onto 96 well plates at a cell density of 1×10^3 cells/well. Untreated FAK-WT SCC cells were also included as positive control. To check whether Axl contributed to cell proliferation, the seeded cells were fixed with 25% trichloroacetic (TCA) acid at day 0 and day 3. The fixed cells were stained with SRB dye for 30 min., after which the excess dye was removed by washing repeatedly with 1% glacial acetic acid. The protein-bound dye was dissolved in a tris-based solution to determine the optical density (OD) at 540 nm using a microplate reader.

As shown in Figure 4.9b, Axl siRNA transfected cells demonstrated no significant difference ($P > 0.05$) in cell proliferation compared to scrambled siRNA treated FAK-WT SCC cells. Therefore this finding indicates that Axl is not required for SCC cell proliferation.

4.1.9 Axl knockdown affects cell polarization of SCC FAK-WT cells

In order to determine whether Axl contributes to cell polarisation, Axl expression was knocked-down using siRNA in FAK-WT cells and then ability of the cells to polarise towards a wound created in a confluent monolayer was analysed.

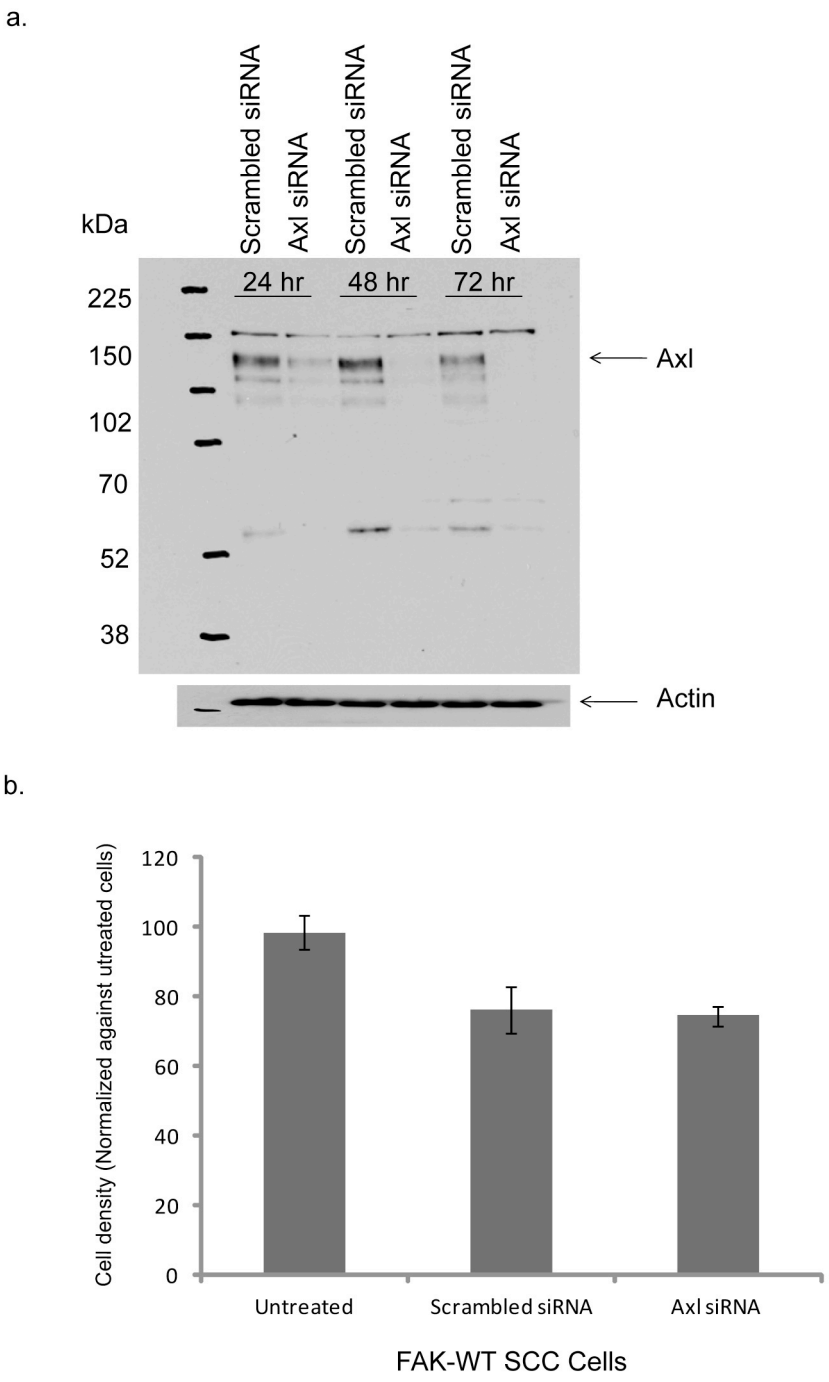


Figure 4.9 Down-regulation of Axl had no effect on SCC cell proliferation

(a) Analysis of siRNA-induced alterations of Axl protein expression by immunoblotting. Protein extracts from FAK-WT cells transfected either scrambled or Axl siRNA were immunoblotted with anti-Axl antibody. Immunoblotting of actin is also shown (lower panel). (b) FAK-WT and FAK-WT cells transfected with either scrambled or Axl siRNA were seeded in 96 well plate. To check whether Axl contributed to cell proliferation, seeded cells were fixed with 25% TCA and then stained for 30 minutes with SRB dissolved in 1% acetic acid. The OD at 540 nm was determined as absorbance value. Data presented are 1 of 3 independent experiments. Error bars represent standard errors of means. Statistical significance was calculated using a student t-test.

The polarised morphology of migrating cells involves the alignment of the Golgi apparatus in front of the nucleus towards the direction of movement. To determine whether Axl was required for cell polarity, scrambled and Axl siRNA transfected FAK-WT SCC cells were analysed using an assay that visualises the reorientation of the Golgi apparatus relative to the nucleus in cells along the wound (Etienne-Manneville and Hall, 2001; Serrels et al., 2010). Cells were plated onto fibronectin (FN) for 2 hours, then wounded and left to polarise for 1.5 hours, before being fixed and stained with anti-GM130 (Golgi marker), nuclear DAPI stain and TRITC phalloidin. At least 100 cells were scored for each three independent experiment. Cells that displayed Golgi in front of the nucleus towards the wound were scored as polarised cells, while cells that had Golgi away from the wound, either on the sides or back of the nucleus, were scored as non-polarised cells, and the percentage of the polarised cells was calculated. We found that the polarisation of Axl siRNA transfected FAK-WT SCC cells was significantly suppressed in contrast to the scrambled siRNA FAK-WT SCC cells, thus demonstrating either an inability to sense the wound or turn towards it (Figure 4.10).

4.1.10 Effect of Axl knockdown on SCC cell spreading

We next investigated whether Axl participates in SCC cell spreading. Cell spreading is characterised by formation of protrusive structures. It has been shown that FAK binds to the receptor for activated C kinase 1 (RACK1) and localises in nascent contacts (Serrels et al., 2010).

Nascent adhesion contacts are defined here as early structures that are formed between cells and extracellular matrix (ECM).

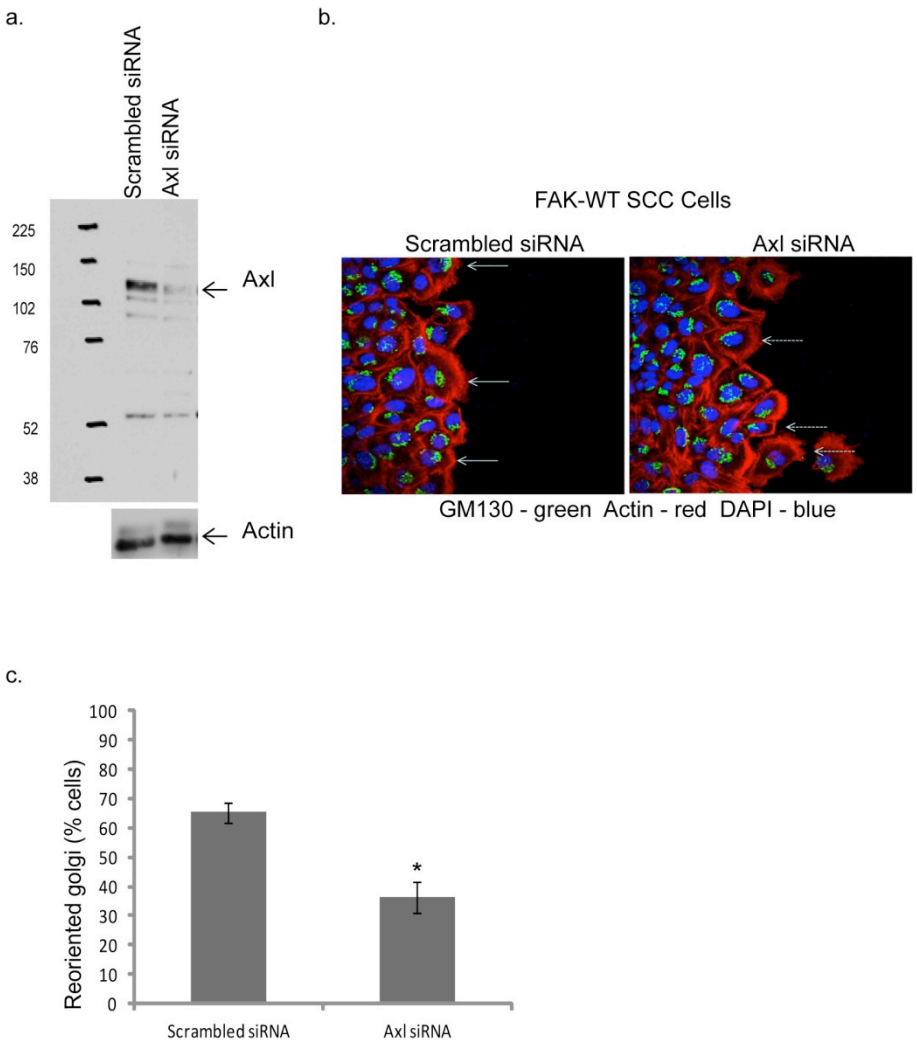


Figure 4.10 Axl knockdown affects cell polarisation of FAK-WT SCC cells

(a) Protein extracts from scrambled or Axl siRNA transfected FAK-WT SCC cells were immunoblotted with anti-Axl and anti-actin antibodies. (b) Scrambled and Axl siRNA transfected FAK-WT SCC cells were plated on FN for 2 hr, wounded, and after 1.5 hr fixed and stained for anti-GM130 (green), TRITC phalloidin (red) and DAPI (blue). Solid arrows (left panel) show cells in which the Golgi is orientated toward the wound, and broken arrows (right panel) indicate cells in which the Golgi is orientated away from wound. (c) Percentage of each cell type with the Golgi orientated to the wound was calculated from a count of 100 cells in each experiment. Columns and error bars represent the mean ± SEM (n=3). Statistical significance was calculated using a student t-test.

Disruption of the interaction between FAK and RACK1 impairs localisation of RACK1 at nascent adhesions structures, and cells become rounded, suggesting that FAK regulates formation of nascent adhesions through its interaction with RACK1. As RACK1 is an early adhesion marker, we decided to examine RACK1 in peripheral protrusive structures in scrambled or Axl siRNA transfected FAK-WT SCC cells using an assay previously described in Serrels et al paper (Serrels et al., 2010).

Essentially, FAK-WT cells transfected with either scrambled or Axl siRNA and suspended in PBS for 1hr at 4^{o}C. The cells were pelleted by centrifugation at 1,000 rpm for 5 min, and re-suspended in SFM before plating onto coverslips covered with FN at a cell density of 2×10^{5} cells/well. The cells were then allowed to spread for 30 minutes before being fixed and stained for actin and RACK1 (Figure 4.11a). At least 100 cells were scored for each three independent experiment. Cells with Rack1 in peripheral protrusive structures were scored as spreading cells, while cells with smooth edges without protrusive structures were scored as rounded cells. The spread cells were counted and quantified and the percentage of the spreading cells were calculated.

The results showed that Axl knockdown cells demonstrate a significant decrease in their ability to spread (Figure 4.11b).

4.1.1 Axl knockdown inhibits chemotactic cancer cell invasion

It has been demonstrated that Axl overexpression is associated with motility and invasiveness in a variety of human cancers.

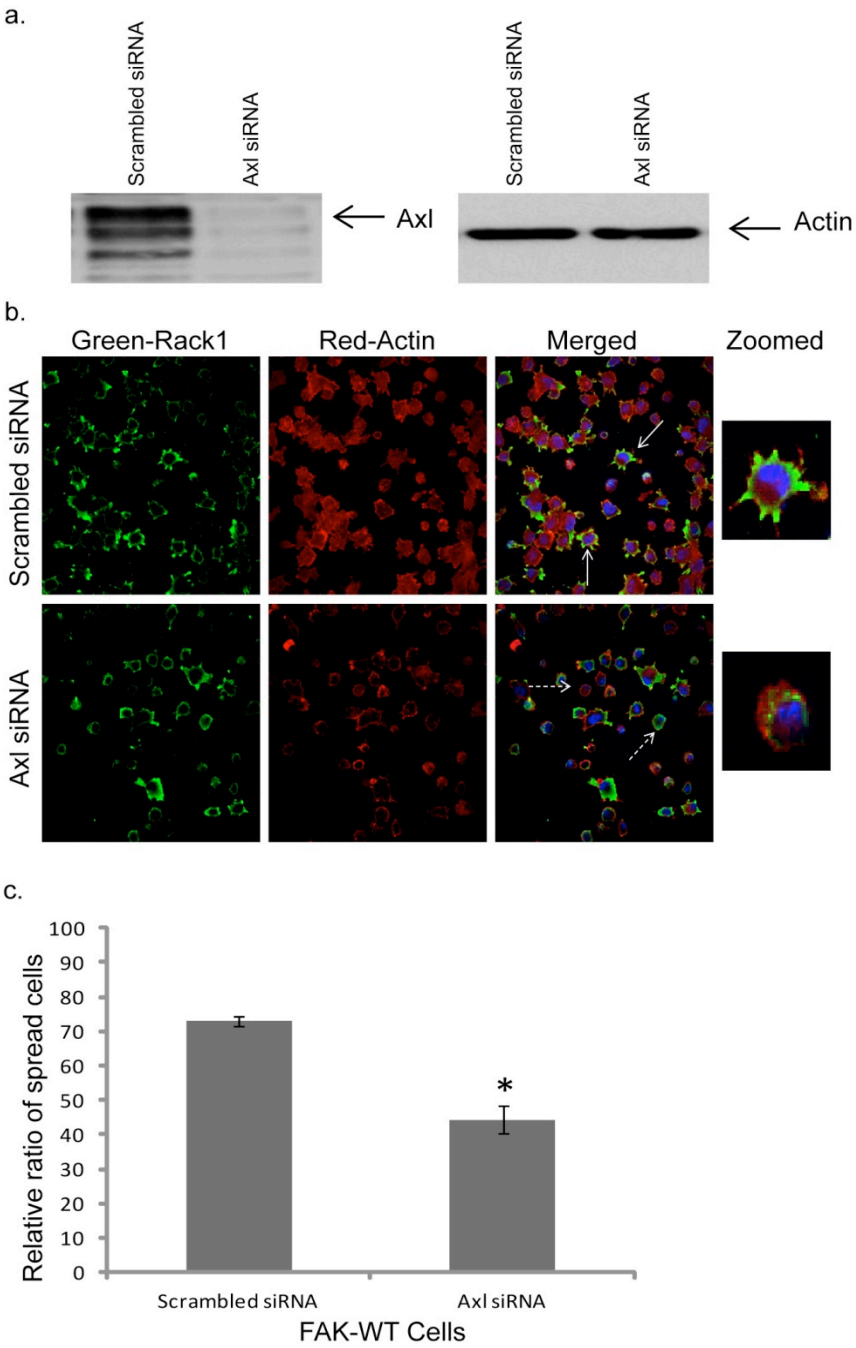


Figure 4.11 Axl knockdown cells demonstrate a significant decrease in their ability to spread

Scrambled or Axl siRNA transfected FAK-WT SCC cells were either (a) lysed and analysed by immunoblotting using anti-Axl and anti-actin antibodies, or (b) suspended in PBS for 1hr at 4^{o}C. After being harvested by centrifugation, the cells were re-suspended in SFM before plating onto FN-coated coverslips. The cells were then allowed to spread for 30 minutes before being fixed and stained for RACK1 (green), actin (red) and DAPI (blue). Solid arrows represent spreading cells, and broken arrows indicate rounded cells (c) Quantification of cell spreading on fibronectin. Columns and error bars represent the mean ± SEM (n=3). Statistical significance was calculated using a student t-test.

Therefore to examine the functional significance of Axl expression in SCC cell motility and invasiveness, stable Axl knockdown FAK-WT cells were generated using Axl shRNA retroviral clone in pSM2 backbone. Retrovirus were generated by transfecting Phoenix Eco packaging cells with Lipofectamine 2000 for 24 hours followed by collection of virus containing supernatant in SCC media containing 20% FBS. FAK-WT SCC cells were then infected with filtered virus containing 5µg/ml polybrene. Infection of SCC cells was carried out three times and followed by selection in puromycin. FAK-WT SCC cells expressing scrambled shRNA were also generated. SCC cells were then analysed for knockdown efficiency by western blotting (Figure 4.12a).

FAK-WT cells expressing either scrambled shRNA or Axl shRNA were seeded onto transwell filters and allowed to invade through the Matrigel towards a chemotactic stimulus for 72 hours. The cells were then labelled with calcein AM dye and visualised in the Matrigel at 10µm intervals by confocal sectioning (Figure 4.12b), and the percentage of cells invading 60µm into the 3D-gel was quantified (Figure 4.12c). We found that Axl knockdown significantly attenuated the chemotactic invasion of FAK-WT cells. FAK-/- SCC cells also fail to invade through Matrigel (Serrels et al., 2010). We suggest that FAK/Axl interaction may facilitate invasion of SCC cells.

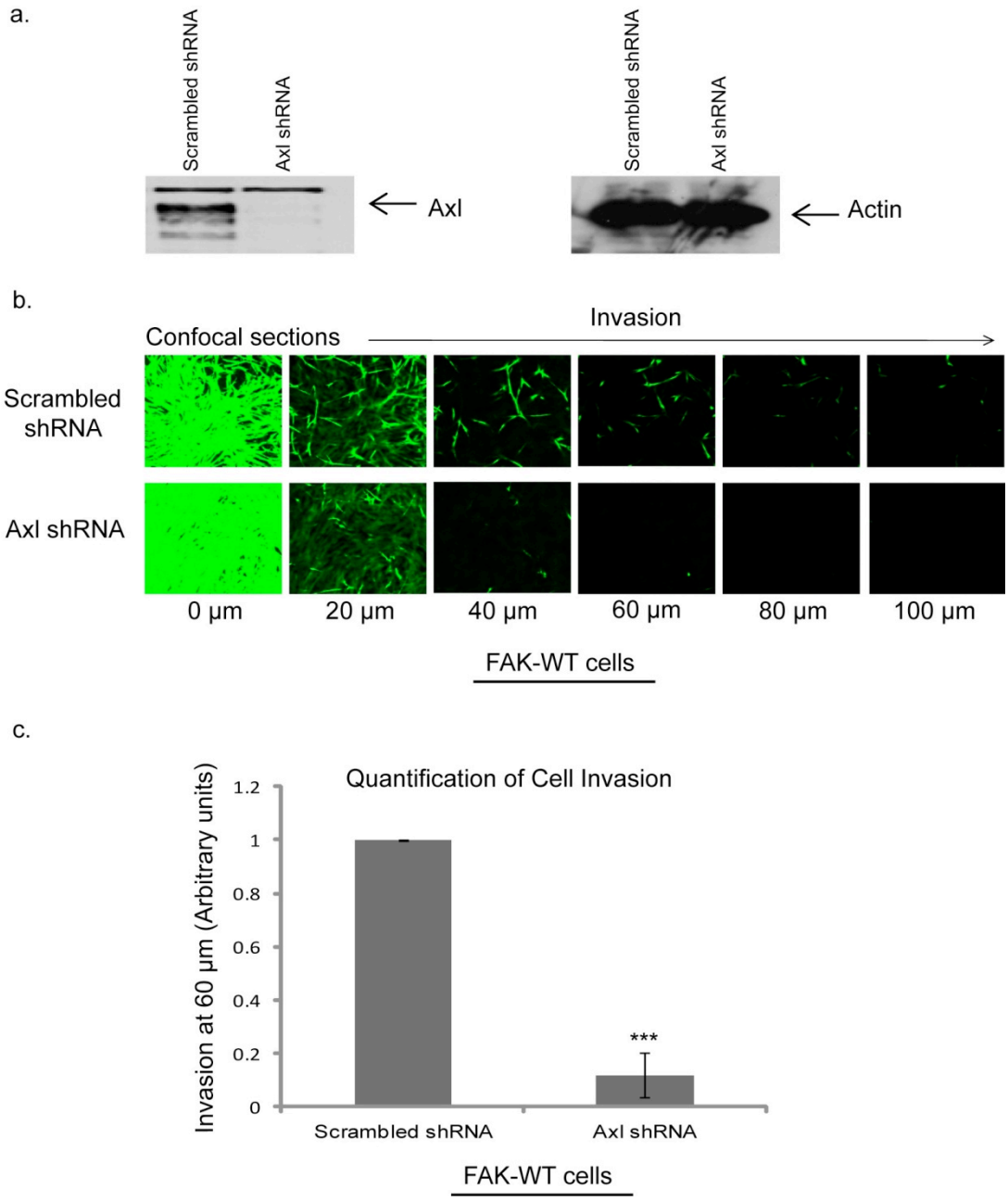


Figure 4.12 Axl knockdown inhibits chemotactic cancer cell invasion

Stable Axl knockdown FAK-WT cells were generated using Axl shRNA retrovirus in pSM2 backbone. Axl knockdown efficiency were analysed by immunoblotting using anti-Axl and anti-actin antibodies. (b) FAK-WT cells with a stably knockdown of Axl were seeded on transwell filters and allowed to invade Matrigel towards a serum gradient. After 72 hours cells were labeled with Calcein AM and visualized by confocal microscopy in the Matrigel at 10µm intervals. Scrambled control cells were also included in the assay. b) Quantitation of results is also shown. The data represents the mean ± SEM of three separate experiments. Statistical significance was calculated using a student t-test. ($p<0.01$)

4.2 Discussion

Axl was originally identified as an oncogene in patients with chronic myeloid leukaemia (CML) (O'Bryan et al., 1991). Its activation has been implicated in various cellular responses, including cell proliferation, migration, adhesion and cell survival [reviewed in (Hafizi and Dahlback, 2006; Lemke, 2013)]. Due to its underlined importance in these processes, we considered that Axl may be an interesting novel binding partner of FAK. Several reports had demonstrated a functional link between Axl expression in tumour growth, migration and metastasis, and association between Axl overexpression and invasiveness and metastasis has been reported for lung (Shieh et al., 2005), prostate (Sainaghi et al., 2005), gastric (Wu et al., 2002) and breast cancers (Meric et al., 2002), as well as renal cell carcinomas (Chung et al., 2003).

The data presented in this study provide strong evidence of the physical interaction between the FAK and Axl proteins in SCC cells, which was demonstrated by co-immunoprecipitation and pulldown experiments. This verification is very important for associations found using protein array approaches, demonstrating that the association between FAK and Axl is not an artefact that occurs only in the protein array, but an actual physical interaction occurring in mammalian cells.

In addition, the direct interaction of FAK and Axl was confirmed by in vitro binding assays using purified GST-fusion fragments of individual FAK domains and His-tagged Axl intracellular domain. Our data demonstrate that there is perhaps more than one binding site on FAK; however, the kinase domain binds more robustly. This finding is in line with the fact that the FAKΔ375 recombinant protein used in the

protein array screen contains the kinase domain. FAK's kinase domain mediate interaction has also been reported previously, such as FAK interacts with FIP200 through its kinase domain (Abbi et al., 2002).

To strengthen our finding further I wanted to show whether FAK and Axl proteins co-localise in SCC cells, and if so, where. I attempted several IF experiments with two Axl antibodies from different companies, but these were unsuccessful. Antibodies did not work for IF, which prevented us from identifying any co-localisation.

To activate FAK, an activator protein is required to trigger FAK FERM domain release from the kinase domain before $FAKY^{397}$ can be autophosphorylated (Lietha et al., 2007). To see whether FAK/Axl interaction requires phosphorylated Y397, Src dependent phosphorylation of FAK or FAK's kinase activity, we studied association between FAK and Axl using FAK-/- SCC cells expressing different FAK mutants. This study demonstrated that FAK/Axl interaction is not dependent on phosphorylation of FAKY397, Src dependent phosphorylation sites or FAK's kinase activity, indicating that the FAK/Axl interaction is predominantly a scaffolding function of FAK and is not modulated by any of the major phosphorylation events characterised for FAK.

Several studies have previously suggested that FAK is phosphorylated on tyrosine and serine residues by diverse kinases after cell activation, including the RET receptor tyrosine kinase, which directly phosphorylates FAK on Y576/Y577 and Y925 (Plaza-Menacho et al., 2011), ERK, which phosphorylates FAK at S910. The latter creates a binding site for PIN1 (Zheng et al., 2009), and Rho-dependent kinase

(ROCK), which directly phosphorylates FAK on S732 (Le Boeuf et al., 2006). Therefore, to examine the potential role of Axl in the process of FAK phosphorylation, we performed an immunoblotting analysis using phospho-specific antibodies for FAK. Our data suggest that Axl does not regulate the phosphorylation of FAK at indicated tyrosine residues (Figure 4.8).

Using an antibody directed against the intracellular portion of Axl, we detected not only high levels of full length receptor, but also higher level of the lower molecular weight (~55 kDa) protein (Figure 4.3b), that I assume that it is the cleaved intracellular fragment of Axl. Other Receptor Tyrosine Kinases (RTKs) have been reported to undergo similar proteolytic cleavage processes to release a soluble extracellular domain as well as a truncated kinase domain, including MET and CSF-1R. The exact role of proteolytic cleavage in RTK function is unclear; however, it has been shown that soluble Axl (sAxl) generated by proteolytic cleavage mediated by disintegrin-like metalloproteinase 10 (ADAM10) plays a role in promoting cell migration and activation of membrane bound full-length Axl (Budagian et al., 2005).

Pronounced Axl expression has been documented in a variety of cancers, where it has been shown to be crucial for maintaining tumour cell proliferation. For example, downregulation of Axl using a monoclonal antibody attenuates the growth of non-small cell lung carcinoma xenografts by reducing cell proliferation and inducing apoptosis (Li et al., 2009). Similarly, Axl knockdown inhibits proliferation and induces apoptosis of osteosarcoma cells through downregulation of the Akt pathway (Zhang et al., 2013). However, Axl overexpression has no effect on the cell proliferation of bladder cancer cells (Sayan et al., 2012), suggesting that Axl is

required for cell proliferation only in some cancer types. The data we present here also show that Axl is not required for SCC cell proliferation in SCC cells *in vitro*.

Axl has also been shown to regulate cancer cell migration. In breast cancer models, inhibition of Axl signalling by a dominant-negative Axl mutant, or by short hairpin RNA knockdown of Axl, decreased highly invasive breast cancer cell motility (Zhang et al., 2008). For cells to migrate a front-rear polarity axis must first be generated. Defects in cell polarity have been shown to be associated with tumorigenesis and metastasis. In order to determine whether Axl contributes to SCC cell polarity, we measured the reorientation of the Golgi apparatus relative to the nucleus in cells along the wound. After knocking down Axl protein expression, cells demonstrated a significant disruption of polarisation. This may have resulted from an inability to sense the wound or turn towards it. We also investigated the role of Axl on SCC cell spreading, which might contribute to cell migration. The data show that Axl knockdown cells demonstrate a significant decrease in their ability to spread. Based on our data we can conclude that Axl deficient SCC cells display impairment of the cell spreading and cell polarity required for migration.

In addition to cell proliferation and migration, Axl overexpression has also been linked to cancer invasion. Axl knockdown blocks the invasive phenotype of breast cancer cells (Holland et al., 2010). It also regulates invasiveness in lung (Shieh et al., 2005), pancreatic (Koorstra et al., 2009) and gastric (Sawabu et al., 2007) carcinomas. Moreover, the involvement of Axl in promoting cancer cell migration and invasion has also been demonstrated *in vitro*: overexpression of Axl in the weakly invasive breast cancer cell line MCF7 endowed the cells with a highly invasive phenotype (Zhang et al., 2008). Here, we identified that Axl knockdown

significantly attenuated the chemotactic invasion of SCC-FAK-WT cells. As Axl expression is also upregulated in this cell line, compared to normal skin keratinocytes, our data further imply an important role for Axl in invasiveness of SCC cells. FAK has also been shown to be critical for SCC cell invasion: FAK deficient cells did not invade at all relative to FAK-wt-expressing control cells (Serrels et al., 2010). Furthermore, it appears that both proteins (FAK and Axl) can regulate cancer cell invasion by a similar pathway – MMP-mediated matrix degradation (Canel et al., 2008; Tai et al., 2008). We therefore suggest that the FAK/Axl interaction may confer invasive potential on SCC cells. However, the possibility that Axl invasive activity in SCC cells is independent of FAK cannot be excluded. Therefore, FAK-Axl interaction requires further investigation that will be described in future work. However, using FAK as a target binding protein, I have identified Axl as a protein that is upregulated in SCC cells and mediates polarisation, cell spreading and invasion *in vitro*.

Summary

The main findings of this study are:

1. We found a novel FAK binding partner, Axl.
2. It appears that the FAK/Axl interaction is predominantly a scaffolding function of FAK that seems to be unregulated, at least by any of the major phosphorylation events characterised for FAK.
3. Axl is not involved in the phosphorylation process of FAK.
4. Axl has no effect on SCC cell proliferation; however, Axl knockdown resulted in a significantly impaired ability of SCC cell to spread or polarise.

5. Finally, Axl knockdown significantly attenuated the chemotactic invasion of SCC cells.

Future work

The majority of the questions that need to be addressed have arisen from observation concerning the effect of Axl knockdown on SCC cell invasion. While I have identified the phenotype resulting from Axl knockdown in cancer cells in vitro, the mechanism underlying this effect is unknown. Does the interaction between Axl and FAK contribute to regulation of SCC cell invasion? And if so, how do they contribute to it?

A number of tools are available with which to address these questions:

1. Map binding sites using peptide array analysis. This would enable us to generate an effector mutant FAK protein that cannot bind to Axl. This could be used to determine the role of the FAK/Axl interaction in SCC cell invasion.
2. As both proteins regulate cancer cell invasion mainly by the MMP-mediated matrix degradation pathway, it would be interesting to measure the expression levels of MMPs, including MT1-MMP, MMP-2 and MMP-9, in Axl knockdown SCC cells.
3. There is evidence indicating that sometimes, even when MMP expression is induced, the cells still did not invade or degrade the collagen matrix, or that they show little invasive phenotype unless they were treated with HGF, suggesting that MMP activity may be regulated post-translationally (Hotary

et al., 2000). Therefore matrix degradation assays would provide information on the ability of cells to degrade the collagen matrix.

4. Because epithelial cells are in contact with the ECM only at the basal surface, and in order for cells to degrade the ECM, it is essential that they localise MMPs to the basal membrane. Therefore, it would be interesting to look at localisation of MMPs in Axl knockdown SCC cells.

5. A number of Axl 'inhibitors' are available, including YW327.6S2 and R-428 [reviewed in (Verma et al., 2011)]. YW327.6S2 is a monoclonal antibody that recognises and binds to both human and mouse Axl protein, and inhibits tumour growth in breast cancer mouse model (Ye et al., 2010b). R-428 is a small molecular inhibitor. Previous studies have shown that it inhibits tumour cell metastasis in mouse models of breast cancer (Holland et al., 2010). Therefore, R-428 Axl inhibitors can be used to determine the importance of Axl in SCC cell invasion and metastasis *in vivo*.

Chapter 5

The interaction between FAK and Ambra1

5 The interaction between FAK and Ambra1

5.1 Results

5.1.1 Identification of Ambra1 peptide binding sequences within FAK

To test the interaction between FAK and Ambra1, and to map the sites of Ambra1 binding on FAK, we first performed peptide array binding analysis in collaboration with Dr George Baillie, University of Glasgow.

To do this full length Ambra1 (which was a gift from Prof Cecconi, Rome) was used to generate different Ambra1 mutants: F1, F2 and F3 (Fimia et al., 2007). The F1 mutant represents amino-terminus of Ambra1 containing amino acids 1-532, F2 includes the central region of Ambra1, spanning amino acids from 533 to 751, and F3 is the carboxy-terminus region contains amino acids from 767 to end (767-1269) (Fimia et al., 2007). These were sub-cloned in frame in pGex-4T-3 vector. GST-fusion proteins were then expressed in bacteria and purified, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie Blue (Figure 5.1). Recombinant protein concentrations were determined by comparison with BSA protein.

A scanning peptide array of full-length FAK was generated as a library of overlapping 25-mer peptides; each sequence was displaced 5 amino acids towards the carboxy-terminus. This immobilised peptide array was incubated with either purified GST or purified recombinant GST-Ambra1-F1, F2 or F3, then probed with anti-GST antibody. Arrays incubated with GST, GST-Ambra1-F1 or GST-Ambra1-F2 were blank as was the array probed with GST antibody alone.

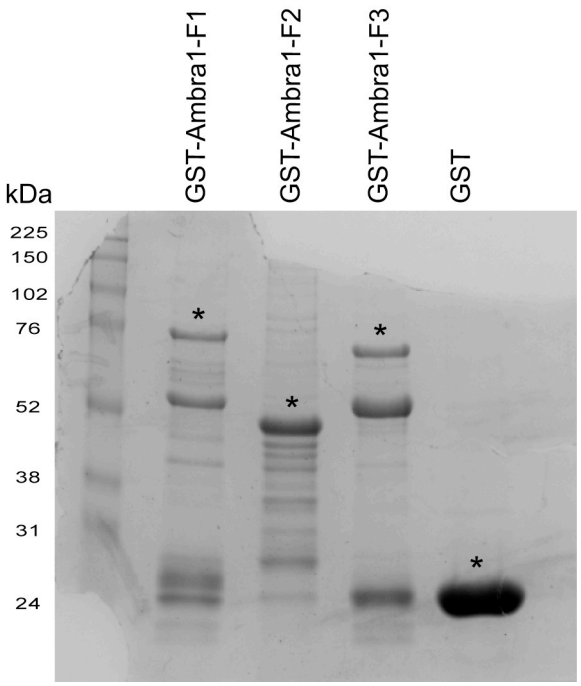


Figure 5.1 Generation of GST-fusion recombinant Ambra1 mutants

Fusion recombinant proteins produced in bacteria were separated by SDS-PAGE and stained with Coomassie blue. GST-Ambra1-F1, F2 and F3 and GST control recombinant proteins are marked with stars.

We did however detect positive interactions on the FAK array probed with GST-Ambra1-F3 signifying a possible interaction with this region of Ambra1 (Figure 5.2).

Alanine scanning peptide arrays were then generated correlating to the peptides that were reacted with antibody in Figure 5.2 by substituting each residue in turn to alanine (A) to assess the contribution of each residue to Ambra1 binding. We showed that in the carboxy-terminal region of FAK (Figure 5.3) these positive interactions disappeared when a proline (P) at residue 875 or at residue 881 was substituted to alanine (Figure 5.3, solid arrows, mutation shown in red). This data suggests that P875 and P881 in the C-terminal of FAK may be binding to the F3 region of Ambra1 and that mutation of these residues might interfere with this interaction.

5.1.2 FAK interacts with Ambra1 in SCC cells

To further confirm the interaction between FAK and Ambra1 in cells, we performed co-IP experiments in SCC cells. FAK-/- and FAK-WT cells were lysed, and immunoprecipitation carried out using anti-Ambra1 antibody (from Sigma). Samples were separated by SDS-PAGE and analysed by immunoblotting using anti-Ambra1 and anti-FAK antibodies. We found that Ambra1 associates with FAK in FAK-WT cells, but not in FAK-/- cells as expected. In contrast, co-IP with control IgG did not bind to FAK (Figure 5.4a). There appeared to be multiple species of Ambra1 recognized by the anti-Ambra1 antibody, which we considered might be different isoforms of the Ambra1 protein, or differently modified versions.

FAK-/- and FAK-WT SCC cell lysates were also incubated with anti-FAK antibody and samples immunoblotted with both anti-FAK and anti-Ambra1 antibodies. We found that FAK forms complex with the upper species of Ambra1 (Figure 5.4b).

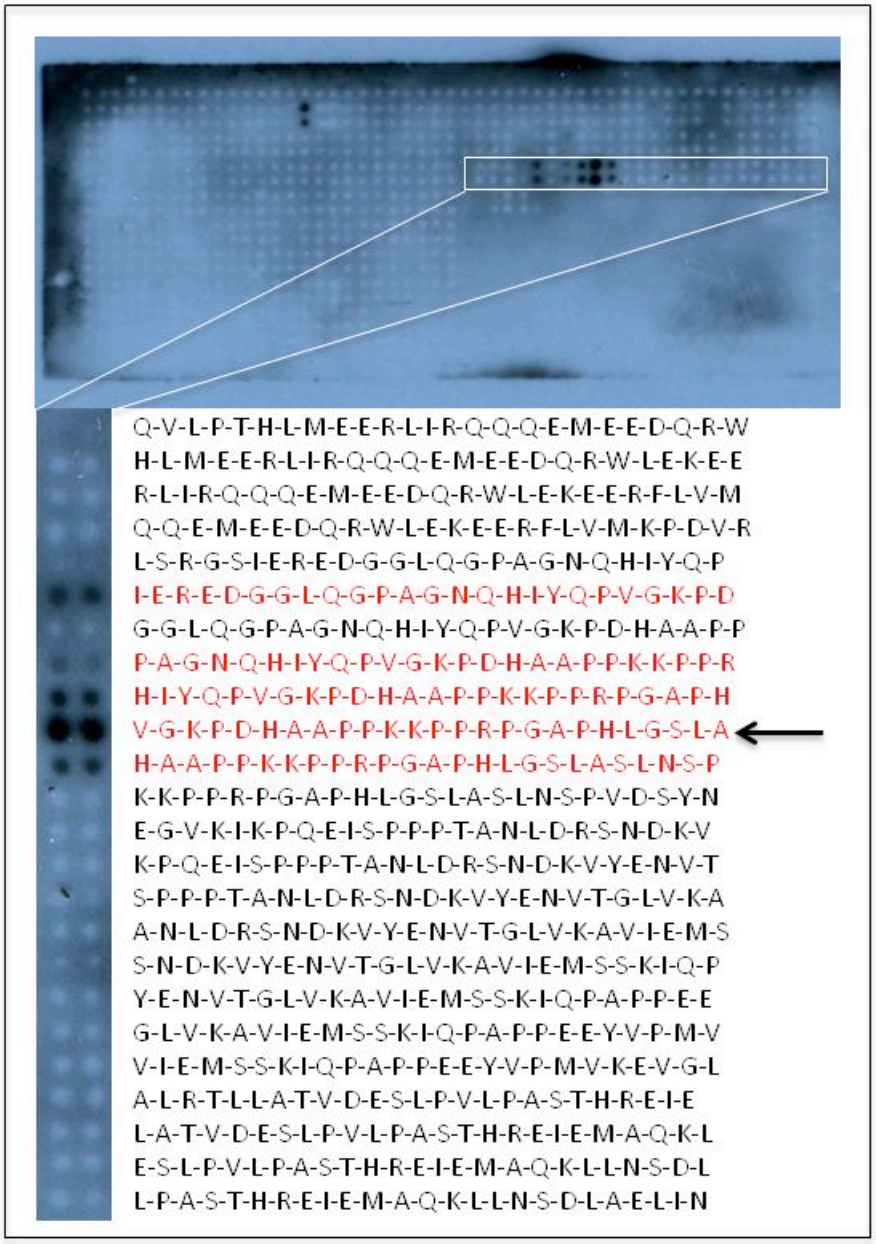


Figure 5.2 Probing a FAK peptide array with GST-Ambra1-F3

Immobilized peptide 'spots' of overlapping 25-mer peptides each shifted along by five amino acids in the entire FAK sequence were incubated with GST-Ambra1-F3 and then probed with anti-GST antibody. Black dots represent a positive interaction between Ambra1-F3 and FAK peptides, indicating potential interaction sites. Data highlights a previously known proline rich region 2 (PRR2) present in the carboxy-terminal region of FAK.

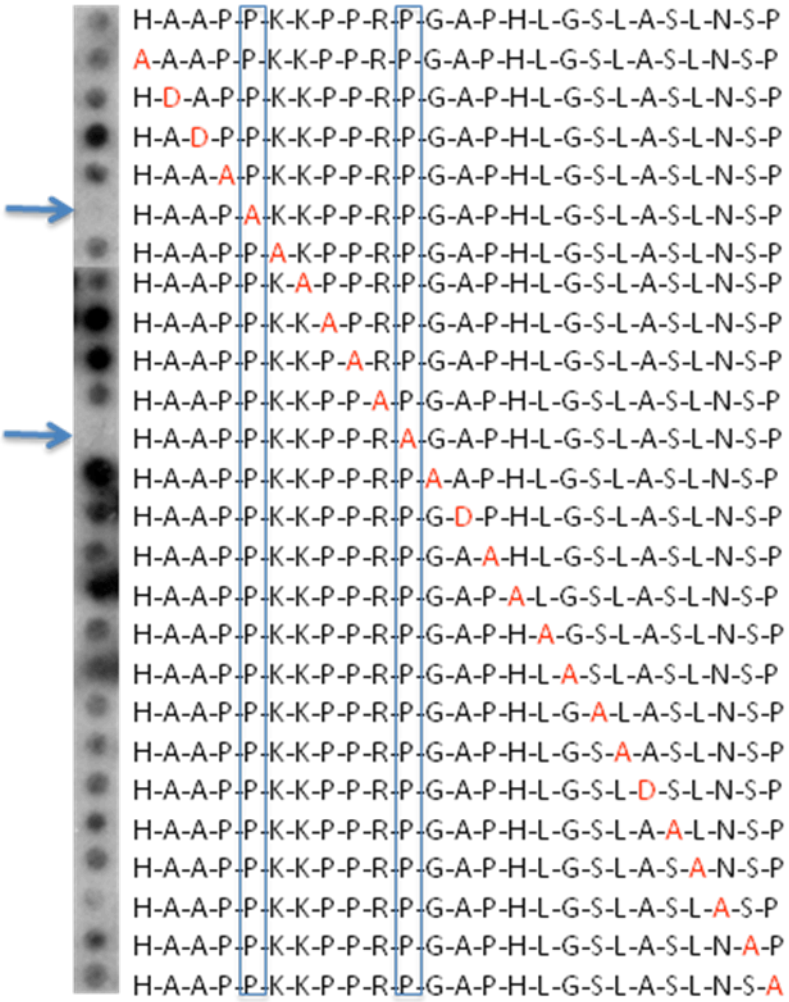


Figure 5.3 Alanine-scanning substitution analysis to probe the binding sites for GST-Ambra1-F3 in the carboxy-terminal region of FAK

Each indicated amino acid of 25-mer peptide representing amino acids 271-295 (H271-P295) of C-terminal region of FAK, were sequentially and individually replaced by alanine (A) or aspartate (D) (shown in red) and probed for GST-Ambra1-F3. 'Dark spots' disappeared (indicated by solid arrows) when P875 or P881 was substituted to A.

These immunoprecipitation experiments clearly demonstrate that FAK interacts with Ambra1 in SCC cells.

Using a confocal microscopy, next we examined the subcellular localisation of Ambra1 and FAK in both FAK-/- and FAK-WT cells. Cells were plated on cover slips and then fixed and stained with anti-FAK (in red) and anti-Ambra1 (in green) antibodies (Figure 5.4c). This shows that FAK localises to focal adhesions in FAK-WT cells (upper left panel), but it is absent in FAK-/- cells (lower left panel), as expected. Ambra1 localises mainly in cytoplasm with some staining evident at the plasma membrane where FAK is also present (solid arrows indicate plasma membrane localization of Ambra1, right panels). However, staining of Ambra1 implied it was predominantly cytoplasmic.

5.1.3 Ambra1 is present in Src-containing autophagosomes in FAK-/- SCC cells

In FAK-deficient cells Src is targeted to autophagosomes (Sandilands et al., 2012a), and as Ambra1 has been shown to influence autophagy in neural systems (Fimia et al., 2007), I wanted to know if it bound to Src and was involved in Src-selective autophagy in FAK-deficient cells.

To investigate whether Ambra1 was in a complex with active Src, FAK-/- and FAK-WT cell lysates were immunoprecipitated with anti-p-Src-Y416 antibody and then probed with anti-Ambra1 and anti-p-Src-Y416 antibodies (Figure 5.5a). We detected an interaction between Src and Ambra1 in FAK-WT cells and found that this interaction was increased in the absence of FAK. We also found that unlike FAK, Src forms complex with several species with molecular weights of ~85 kDa and 130 kDa. They are in the right molecular weight to possibly be Ambra1.

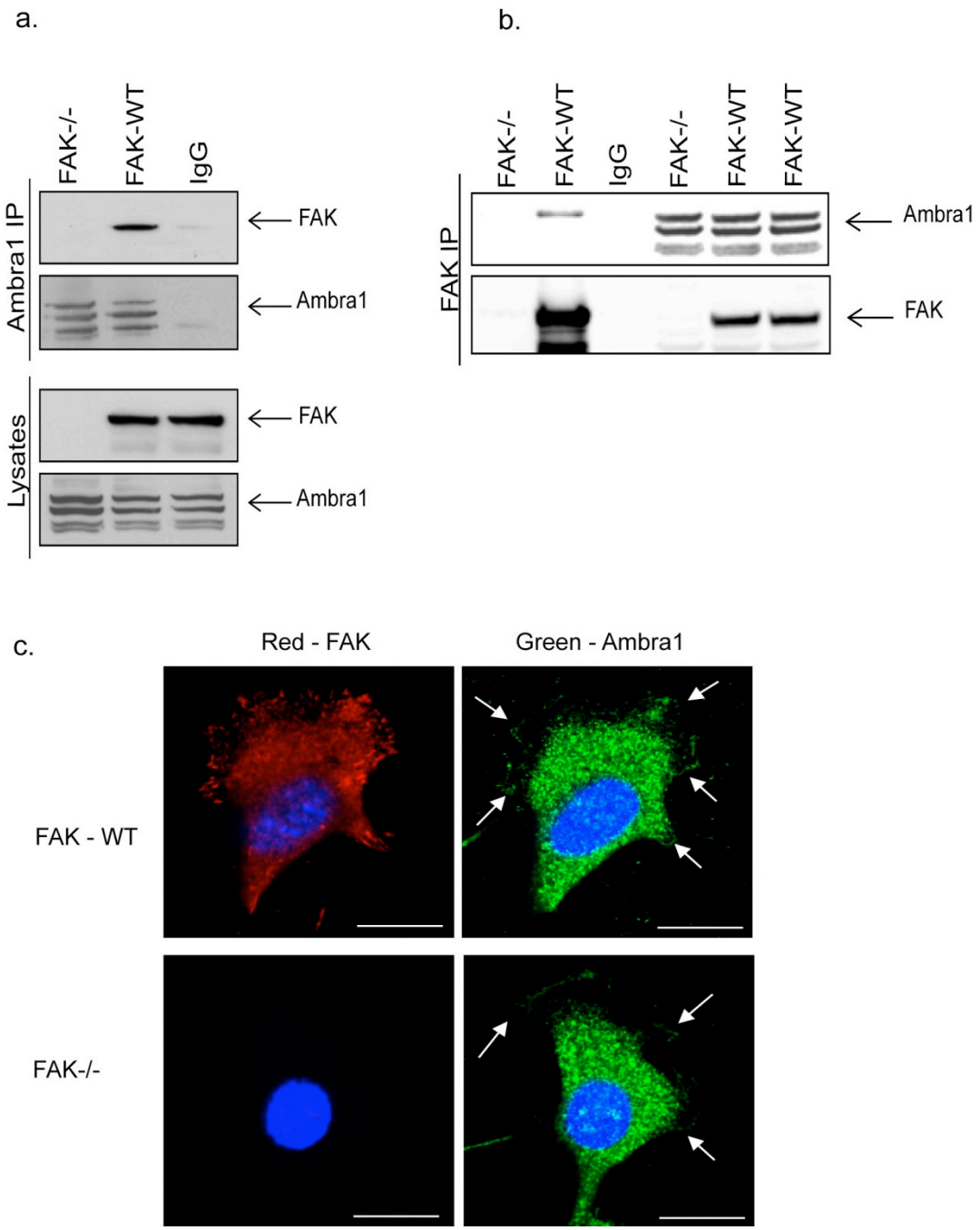


Figure 5.4 FAK interacts with Ambra1 in SCC cells

Cell lysates from FAK-/- and FAK-WT cells were immunoprecipitated with anti-Ambra1 (a) and anti-FAK (b) antibodies. Purified complexes and corresponding total cell lysates were separated by SDS-PAGE and immunoblotting performed using anti-Ambra1 and anti-FAK antibodies. (c) FAK-/- and FAK-WT cells were fixed and stained with either anti-FAK (red in left panels) or anti-Ambra1 (green in right panels) and DAPI (blue). Arrows indicate plasma membrane localization of Ambra1. Scale bars, 20um.

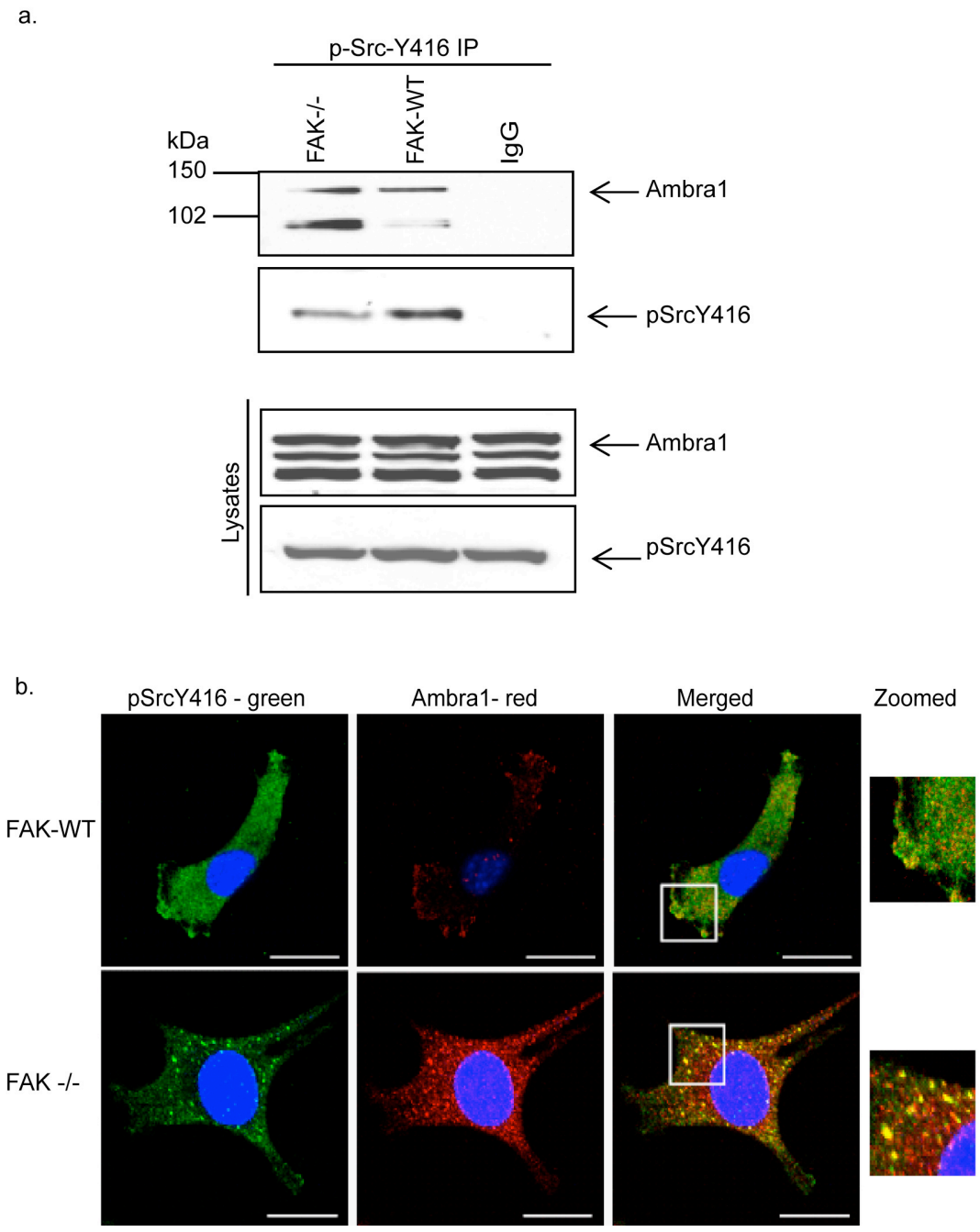


Figure 5.5 Ambra1is present in Src-containing autophagosomes in FAK-/- SCC cells

(a) Cell lysates from FAK-/- or FAK-WT cells were immunoprecipitated with anti-p-Src-Y416 antibody. Purified complexes and corresponding total cell lysates were separated by SDS-PAGE and immunoblotted using anti-Ambra1 and anti-p-Src-Y416 antibodies. IgG control is also included. (b) FAK-WT (upper panel) and FAK-/- SCC cells (lower panel) were fixed and stained with anti-p-Src-Y416 (green), anti-Ambra1 (red) and DAPI (blue). Higher magnification images of the outlined areas are also shown. Scale bars, 20um.

Next we examined co-localisation between p-Src and Ambra1 in FAK-/- and FAK-WT cells. Cells were fixed and stained for Ambra1 (in red), anti-p-Src-Y416 (in green) and DAPI (in blue) (Figure 5.5b). The results show that Ambra1 and anti-p-Src-Y416 co-localise at focal adhesions at the cell edges in FAK-WT cells, and in cytoplasmic puncta in the cytoplasm in FAK-/- cells. These data imply that Ambra1 is also present in Src-containing autophagosomes in SCC cancer cells that are devoid of FAK, and raise the possibility that some Ambra1 is localised to adhesions when FAK is present, but is co-targeted to autophagosomes with Src upon loss of FAK.

5.1.4 Characterization of Ambra1 proficient and deficient MEFs

Around this time as I was carrying out this work, we were able to obtain Ras transformed MEFs with genetic deletion of Ambra1 from Dr Guillermo Velasco (Madrid).

To confirm that these cells were indeed Ambra1 deficient, we first performed RT-PCR. Total RNA was isolated from Ambra1+/+ and Ambra1-/- MEFs, cDNA generated and then PCR performed using specific primers for Ambra1, and for a housekeeping gene beta-2-microglobulin (B2M) (Figure 5.6a). The results confirmed that Ambra1 transcript was present in Ambra1+/+ but not in Ambra1-/- MEFs and that control B2M was present in both, at the same level.

We next prepared cell lysates from these MEFs and from SCC cells and immunoblotted using anti-Ambra1 and anti-actin antibodies to examine the expression level of Ambra1 protein (Figure 5.6b). When we analysed the results, we were surprised to see multiple species in all samples, including Ambra1-/- MEFs, indicating that the Ambra1 antibody we were using was recognising non-specific

bands instead of Ambra1. We also observed the same intracellular staining pattern in Ambra1+/+ MEFs as in Ambra1-/- MEFs with an anti-Ambra1 antibody from Abcam used in our previous experiments, suggesting that this antibody was also not specifically detecting Ambra1 (Figure 5.6c).

These results were very important and indicated that antibodies were being sold by commercial providers were not recognising Ambra1specifically, and were mis-leading – despite recognising multiple species in the right molecular weight range consistent with these species being Ambra1. This highlighted to me the dangers of assuming that commercially sourced antibodies are specific.

Therefore we decided to discard all the data generated using the anti-Ambra1 antibody from Sigma, and test other commercially available anti-Ambra1 antibodies, defining specificity using the Ambra1-deficient MEFs and repeating key experiments.

5.1.5 Validation of anti-Ambra1 antibody from Millipore

We purchased a supposedly specific anti-Ambra1 antibody from Millipore and examined Ambra1 protein expression in Ambra1+/+, Ambra1-/- MEFs, and FAK-/- and FAK-WT SCC cell lines by Immunoblotting. With this antibody we detected a single band ~150kDa in all samples except Ambra1-/- MEFs as expected (Figure 5.7a).

We also validated this antibody using Ambra1 siRNA to knockdown Ambra1 in SCC cells. FAK-/- SCC cells were transfected with scrambled or Ambra1 siRNA (80nM and 100nM). After 72 hours cells were lysed and immunoblotted with anti-Ambra1 or anti-GAPDH antibodies (Figure 5.7b).

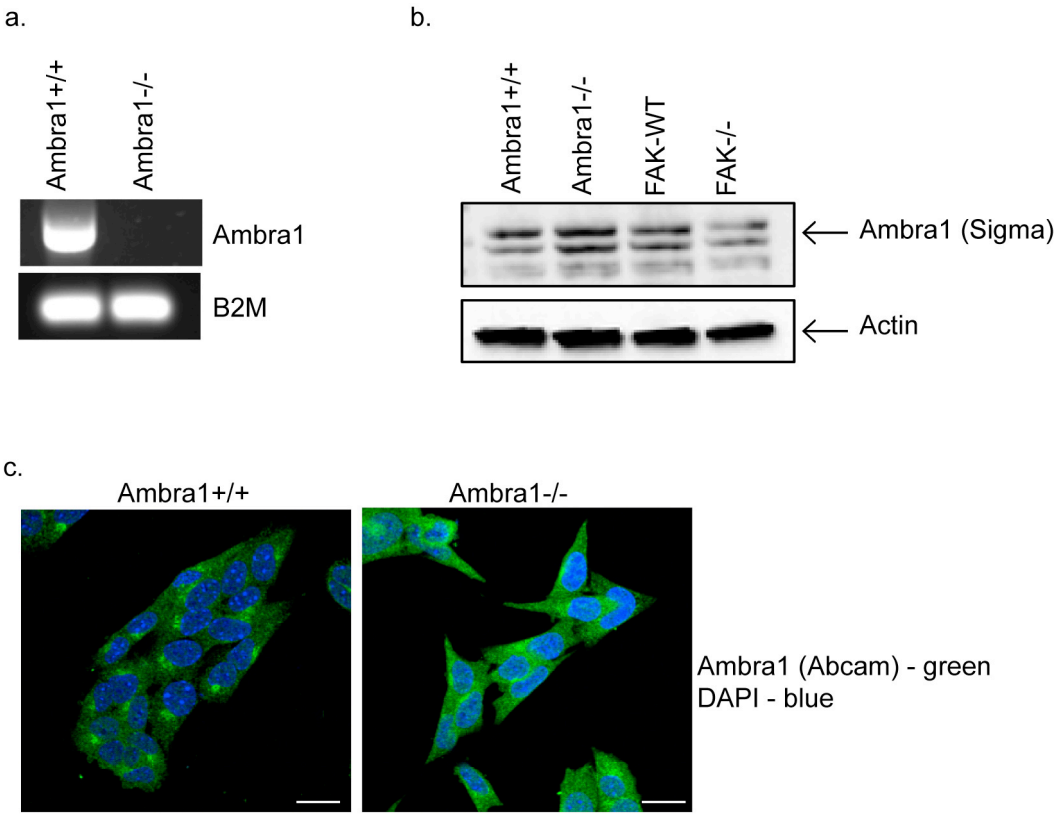


Figure 5.6 characterisation of Ambra1 proficient and Ambra1 deficient MEFs

(a) RT-PCR of Ambra1 and B2M housekeeping gene in Ambra1+/+ or Ambra1-/- MEFs. (b) FAK-WT, FAK-/- SCCs, and Ambra1 +/+ and Ambra1-/- MEFs were lysed, and cell lysates were analysed by immunoblotting using anti-Ambra1 (Sigma) and anti-actin antibodies. (c) Ambra1+/+ and Ambra1-/- MEFs were plated, fixed and stained for Ambra1 (green) and DAPI (blue). Scale bars, 20um.

This data shows that Ambra1 siRNA reduces Ambra1 expression in this cell line, and that the Millipore antibody is specific, and so useful for our studies.

To examine whether the anti-Ambra1 antibody worked for IF, the Ambra1+/+ and Ambra1-/- cells were fixed and stained for Ambra1 and DAPI. We observed that Ambra1 localised to defined punctuate structures in the cytoplasm of Ambra1+/+ cells, which were not detected in Amra1-/- cells. However, we did find staining of Ambra1-deficient MEFs in the cytoplasm, which is presumably non-specific staining.

Taken together, the data above, gave us confidence to carry out further experiments using the anti-Ambra1 antibody obtained from Millipore since it appeared to be specifically detecting Ambra1 in both MEFs and SCC cells.

5.1.6 FAK and Ambra1 interaction

To confirm whether FAK and Ambra1 were associated in SCC cells, co-IP studies using FAK-/- and FAK-WT SCC cells were performed again using the specific Ambra1 antibody. IP of FAK from cell lysate using anti-FAK antibody was followed by immunoblotting with anti-FAK and anti-Ambra1 antibodies (Figure 5.8). This data shows that FAK and Ambra1 do form a complex in these cells.

In the converse experiment, FAK-/- and FAK-WT cell lysates were immunoprecipitated with anti-Ambra1 antibody and then immunoblotted with either anti-Ambra1 or anti-FAK antibodies. These data show that there is an interaction between FAK and Ambra1 in FAK-WT cells and, as expected, co-IP in FAK-/- cells or using IgG control did not result in complex detection (Figure 5.8a and 5.8b).

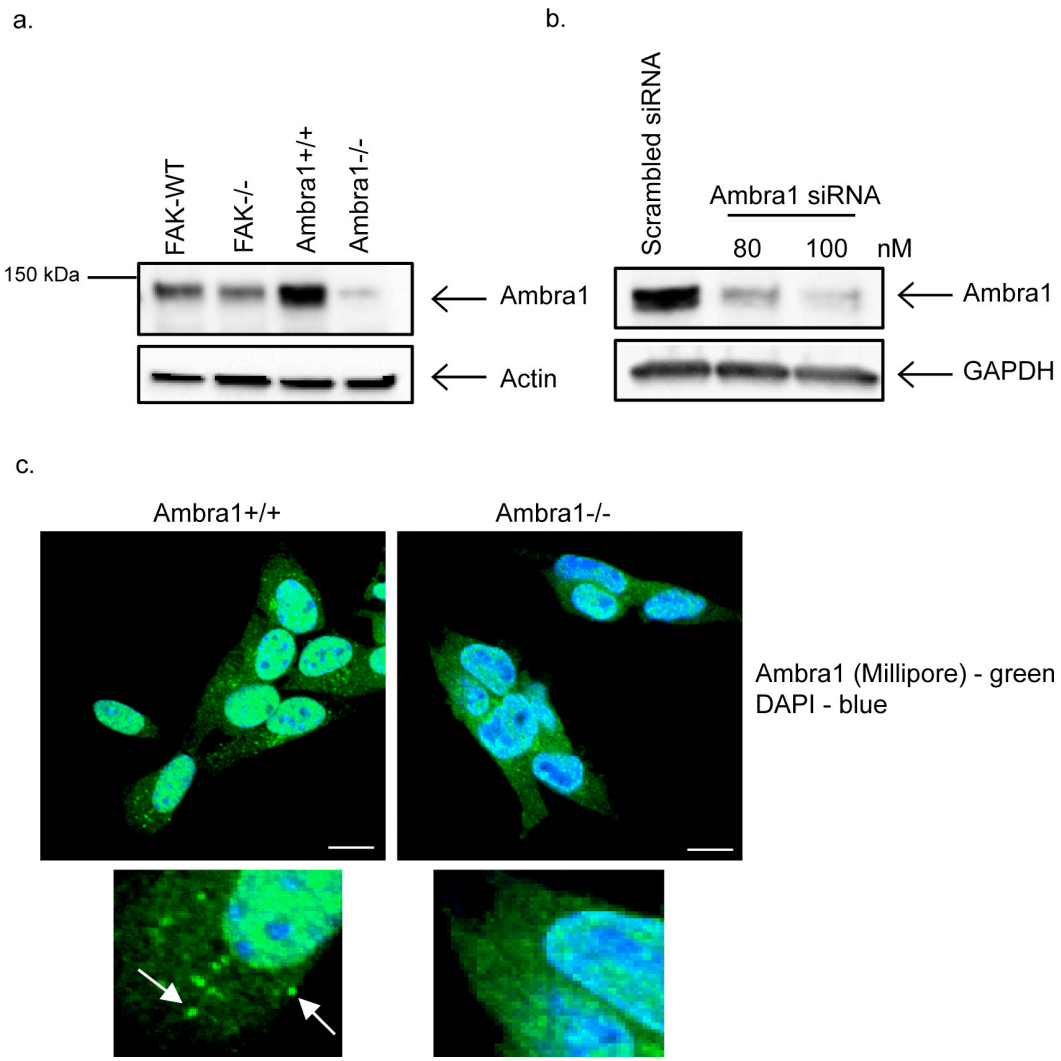
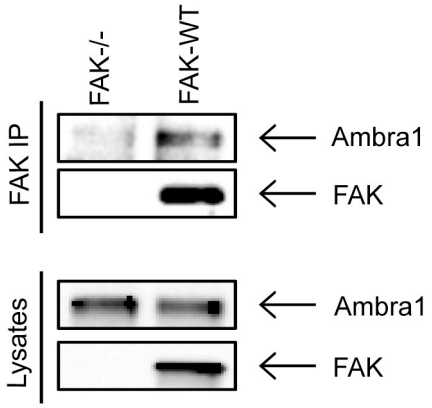


Figure 5.7 Validation of anti-Ambra1 antibody from Millipore

(a) Ambra1 +/+, Ambra1-/- MEFs, and FAK-/- and FAK-WT SCC cells were lysed and subjected to immunoblotting with indicated antibodies. (b) FAK-/- cells were transfected with either scrambled or Ambra1 siRNA. After 72 hours cells were lysed and cell lysates were analysed by immunoblotting using anti-Ambra1 and anti-GAPDH antibodies. (c) Ambra1+/+ and Ambra1-/- cells were plated, fixed and stained for Ambra1 (green) and DAPI (blue). Scale bars, 20um.

a.



b.

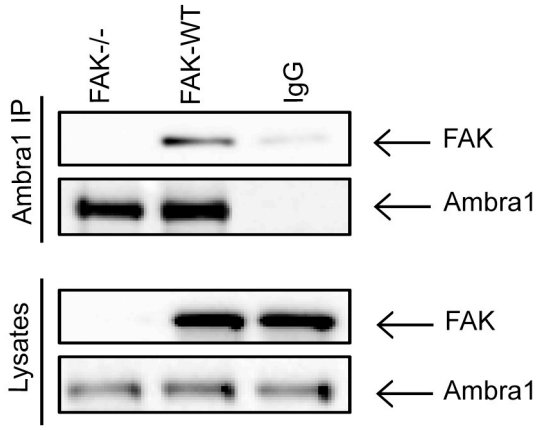


Figure 5.8 FAK interacts with Ambra1 in SCC cells

Cell lysates from FAK-/- or FAK-WT SCC cells were immunoprecipitated with anti-FAK (a) or anti-Ambra1 (b) antibodies. Purified complexes and corresponding total cell lysates were separated by SDS-PAGE and immunoblotted using anti-Ambra1 and anti-FAK antibodies. IgG control was also included.

5.1.7 Regulation of FAK/Ambra1 interaction

To see whether FAK/Ambra1 interaction was regulated by FAK-Y397 phosphorylation, Src dependent phosphorylation of FAK, or by FAK's kinase activity, we used FAK-/- cells stably re-expressing FAK-WT, FAK-Y397F, FAK-Y4F-Y9F and FAK-KD mutants [as used before in (Sandilands et al., 2012b; Serrels et al., 2010)]. Stable expression of the FAK protein was confirmed by immunoblotting (Figure 5.9a). We did not observe any changes in Ambra1 expression, suggesting that this is not regulated by FAK phosphorylation.

To determine whether FAK/Ambra1 interaction was affected in SCC cells expressing these FAK mutants, co-IP experiments were performed. FAK-WT, FAK-Y397F, FAK-Y4F-Y9F, and FAK-KD SCC cells were lysed and then cell lysates were immunoprecipitated with either anti-FAK or anti-Ambra1 antibodies (Figure 5.9c). The immune complexes were then separated by SDS-PAGE and samples immunoblotted for both FAK and Ambra1. The data show that there was a decrease in association between FAK and Ambra1 in FAK-397F, FAK-Y4F-Y9F and FAK-KD expressing SCC cells compared to FAK-WT SCC cells; however the interaction was not completely abolished. Representative quantification of the amount of FAK binding to Ambra1 is shown in Figure 5.9d. However, we were not able to readily quantify the level of Ambra1 binding to FAK since the FAK-Y397F and FAK-Y4F-Y9F expressing SCC cells displayed lower levels of FAK.

5.1.8 FAK binds to Beclin1/PI3KC3 multi-protein complex

In mammals the Beclin1/PI3KC3 containing multi-protein core complex consists of PI3KC3 (Vps34), p150 (Vps15) and Beclin1. Beclin1 is a key regulator of autophagy. It interacts with PI3KC3 in mammalian cells, and regulates generations

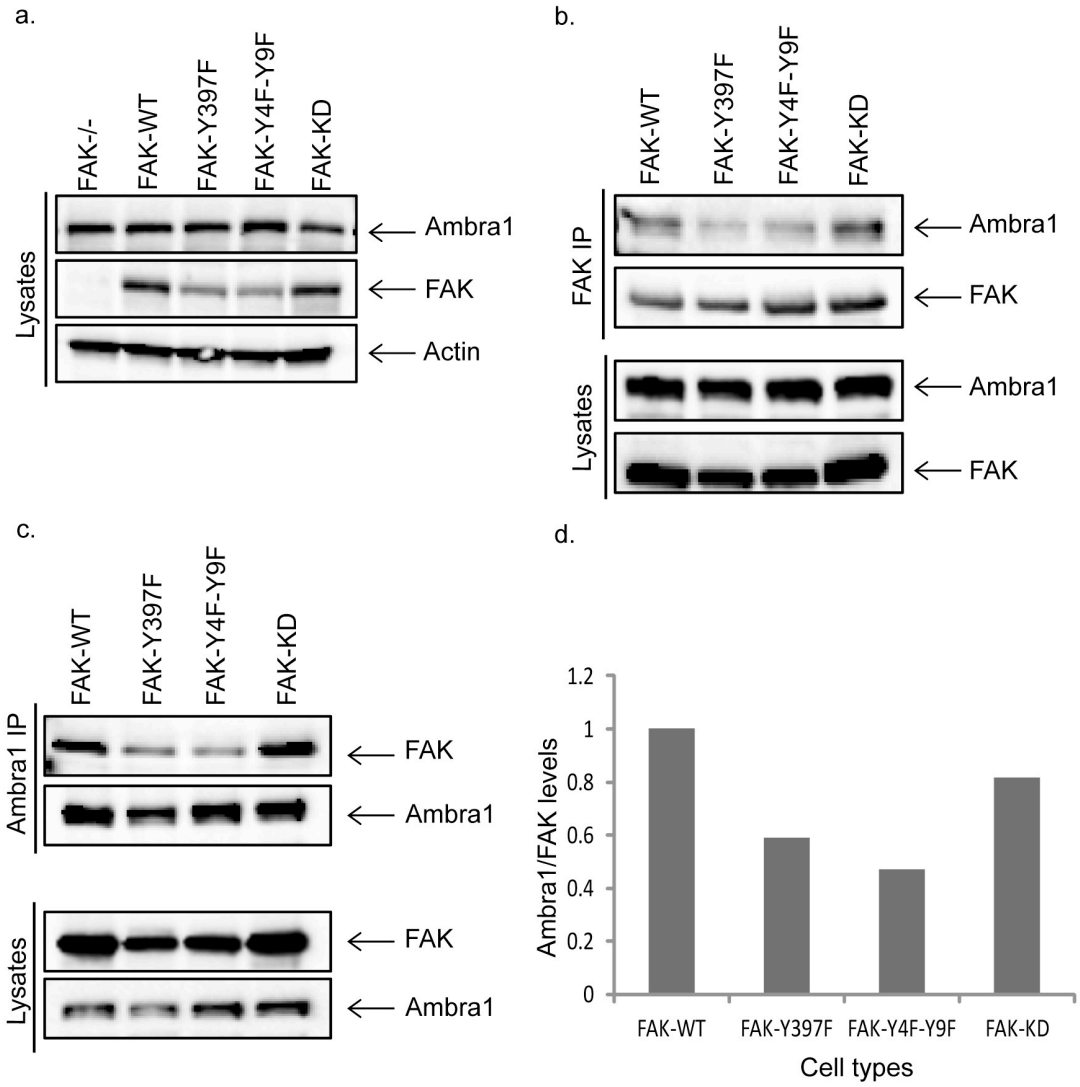


Figure 5.9 Regulation of FAK/Ambra1 interaction

(a) FAK-/-, FAK-WT, FAK-Y397F, FAK-Y4F-Y9F, and FAK-KD SCC cells were lysed and cell lysates were analysed by immunoblotting using anti-Ambra1, anti-FAK and anti-actin antibodies. Lysates from FAK-WT, FAK-Y397F, FAK-Y4F-Y9F, and FAK-KD SCC cells were immunoprecipitated with either anti-FAK (b) or anti-Ambra1 (c) antibodies. The complexes were then analyzed by immunoblotting with anti-FAK and anti-Ambra1 antibodies. Total cell lysates were also directly probed with the same antibodies. c) Representative quantification of one IP experiment is also shown.

of PI3P through PI3KC3-dependent phosphorylation of phosphatidyl inositol (PI) (Furuya et al., 2005; Kihara et al., 2001). PI3P is required for the formation of autophagosomes (Walker et al., 2008).

Several proteins have been identified that interact with the autophagy core complex and positively or negatively regulate PI3KC3 kinase activity, including UV irradiation resistance-associated gene (UVRAG) (Liang et al., 2006), RUN domain and cystein-rich domain containing, Beclin1-interacting protein (Rubicon) (Matsunaga et al., 2009), Bax-interacting factor 1 (Bif-1) (Takahashi et al., 2007) and Ambra1 (Fimia et al., 2007). Since, Ambra1 is a positive regulator of early stages of autophagy as it binds to Beclin1 and promotes Beclin1/PI3KC3 interaction (Fimia et al., 2007), we set out to determine if this complex included FAK, or was regulated by it.

To investigate whether FAK is also a part of the Ambra1/Beclin1/PI3KC3 multi-protein complex we performed co-IP experiments. Lysates from FAK-/- and FAK-WT SCC cells were incubated with anti-PI3KC3 antibody and then immunoblotted with anti-FAK and anti-PI3KC3 antibodies (Figure 5.10a). We observed that FAK immunoprecipitated with anti-PI3KC3 antibody in FAK-WT cells. In contrast, co-IP with control IgG did not precipitate FAK, and there was no complex detected in FAK-/- cells, as expected.

In the converse experiment, FAK-/- and FAK-WT SCC cell lysates were incubated with anti-FAK antibody and then immunoblotted with either anti-FAK or anti-PI3KC3 antibodies (Figure 5.10b). These data confirmed that there is a detectable interaction between FAK and PI3KC3 in FAK-WT cells.

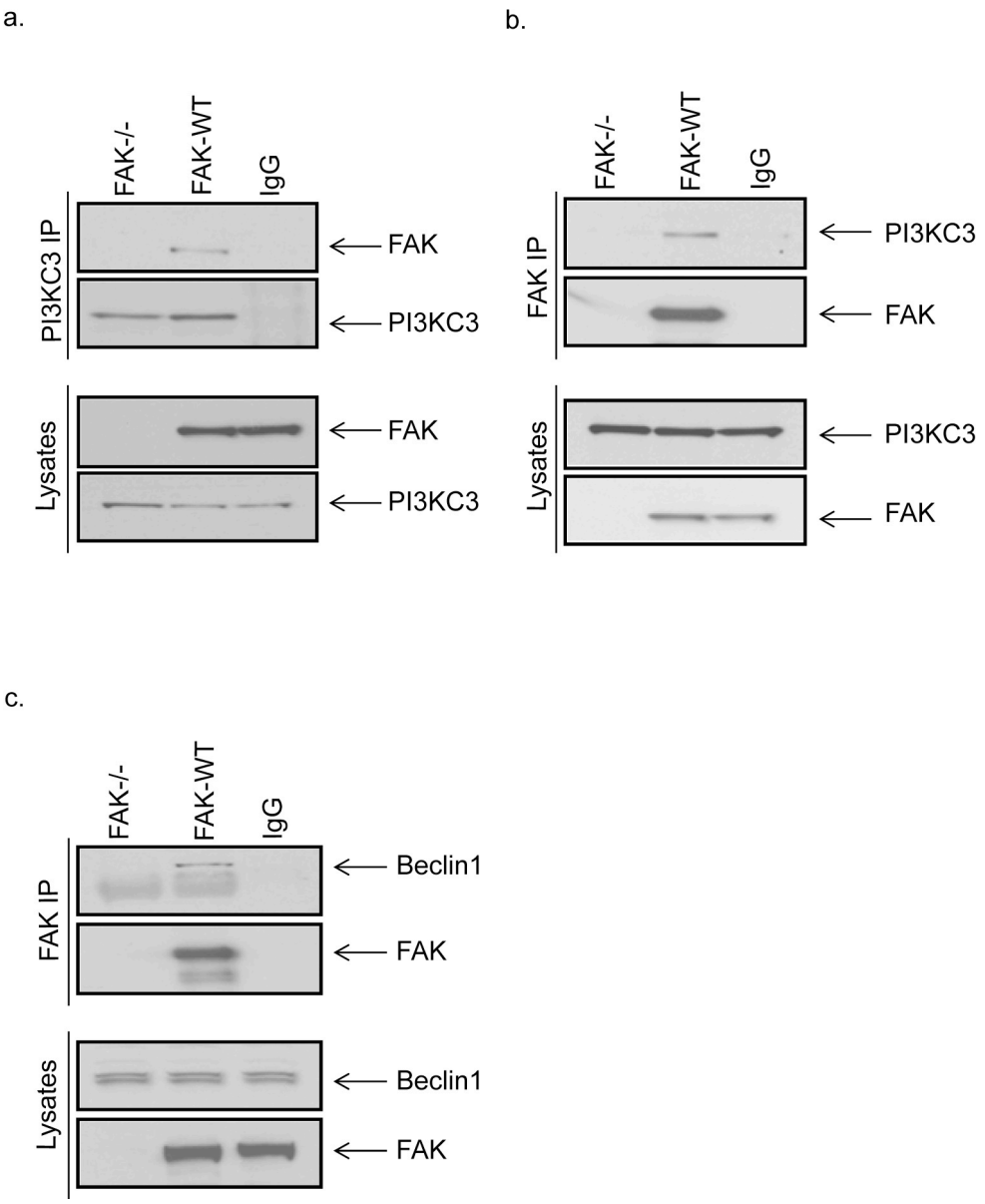


Figure 5.10 FAK interacts with Beclin1/PI3KC3 multi-protein complex

Lysates from FAK-/- and FAK-WT SCC cells were immunoprecipitated with anti-PI3KC3 (a) or anti-FAK (b/c) antibody and probed with anti-FAK, anti-PI3KC3, or anti-Beclin1 antibodies. Cell lysates were also directly probed with the same antibodies. IgG control is also included.

Next we examined whether FAK was in a complex with Beclin1. FAK-/- and FAK-WT SCC cell lysates were immunoprecipitated with anti-FAK antibody and then immunoblotted with anti-FAK or anti-Beclin1 antibodies (Figure 5.10c). Beclin1 was present in a co-IP with FAK in FAK-WT cells, while control IgG did not precipitate Beclin1.

Taken together, we conclude that FAK is part of the Beclin1/PI3KC3 multi-protein complex, although we do not yet know which of these interactions are direct.

5.1.9 FAK is not required for Beclin1/PI3KC3 multi-protein complex formation

We next examined whether FAK had any effect on the complex formation between Beclin1 and PI3KC3 in FAK-/- and FAK-WT SCC cells. Cell lysates were immunoprecipitated with anti-Beclin1 antibody, subjected to SDS-PAGE and then immunoblotted with both anti-Beclin1 and anti-PI3KC3 antibodies (Figure 5.11a). These data show that there is no difference in the interaction between the Beclin1 and PI3KC3 proteins in the presence and absence of FAK.

While PI3KC3 is a cytosolic protein, its autophagosomal membrane association require its interaction with the myristoylated serine/threonine kinase - p150 (Panaretou et al., 1997). The p150 protein is also considered to be a regulatory subunit of PI3KC3 that enhances its kinase activity.

Therefore, we examined the complex between PI3KC3 and p150 proteins in both FAK-/- and FAK-WT SCC cells. Cell lysates were immunoprecipitated with anti-PI3KC3 antibody and then immunoblotted with either anti-p150 or anti-PI3KC3 antibodies (Figure 5.11b). The results show that there is no difference in steady state complex between PI3KC3 and p150 in either the presence or absence of FAK.

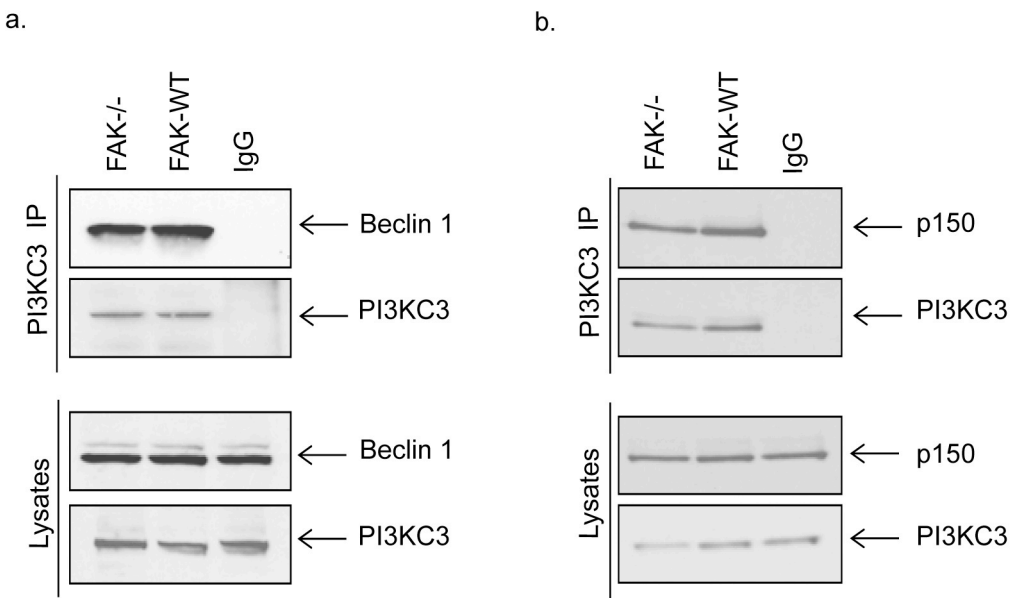


Figure 5.11 Beclin1/PI3KC3 multi-protein complex forms independently of FAK

Lysates from FAK-/- and FAK-WT SCC cells were immunoprecipitated with anti-PI3KC3 antibody and probed with either anti-Beclin 1, and anti-PI3KC3 antibodies (a) or anti-p150, and anti-PI3KC3 antibodies (b). Cell lysates were also directly probed with the same antibodies. IgG control is also included.

Together, these data allow us to conclude that although FAK is part of the Beclin1/PI3KC3 core complex, it is not required for it's formation or maintenance of steady state levels.

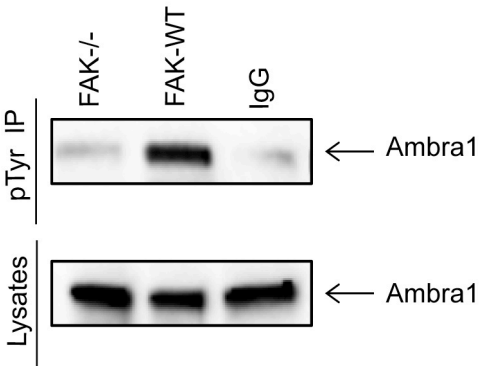
5.1.10 Ambra1 phosphorylation is affected in FAK-/- SCC cells

Next we asked whether FAK is involved in Ambra1 phosphorylation. To test this, we determined the phosphorylation status of endogenous Ambra1 in SCC cells. Lysates from FAK-/- and FAK-WT SCC cells were immunoprecipitated with phosho-Tyrosine (pTyr20) antibody and then immunoblotted with anti-Ambra1 antibody. IgG control was also included (Figure 5.12). The results show, that phosphorylation of Ambra1 is affected in FAK-/- SCC cells, suggesting that FAK is involved in the regulation of phosphorylation of Ambra1. However, we don't know whether FAK directly phosphorylates Ambra1 or does it through another mechanism.

As FAK is a rather weak kinase with only modest kinase activity and only very few examples of FAK-mediated substrate phosphorylation, maybe it is Src that mediates Ambra1 phosphorylation.

5.1.11 Src and Ambra1 interaction

To determine if Src was involved in regulating Ambra1 function, especially as the autophagy regulators Atg7 and LC3 can affect the trafficking of active Src to autophagic puncta in the absence of FAK (Sandilands et al., 2012b), and since Ambra1 has been reported to be important regulator of autophagy (Fimia et al., 2007), we addressed whether it was playing a role in the Src-selective autophagy in FAK-deficient SCC cells.



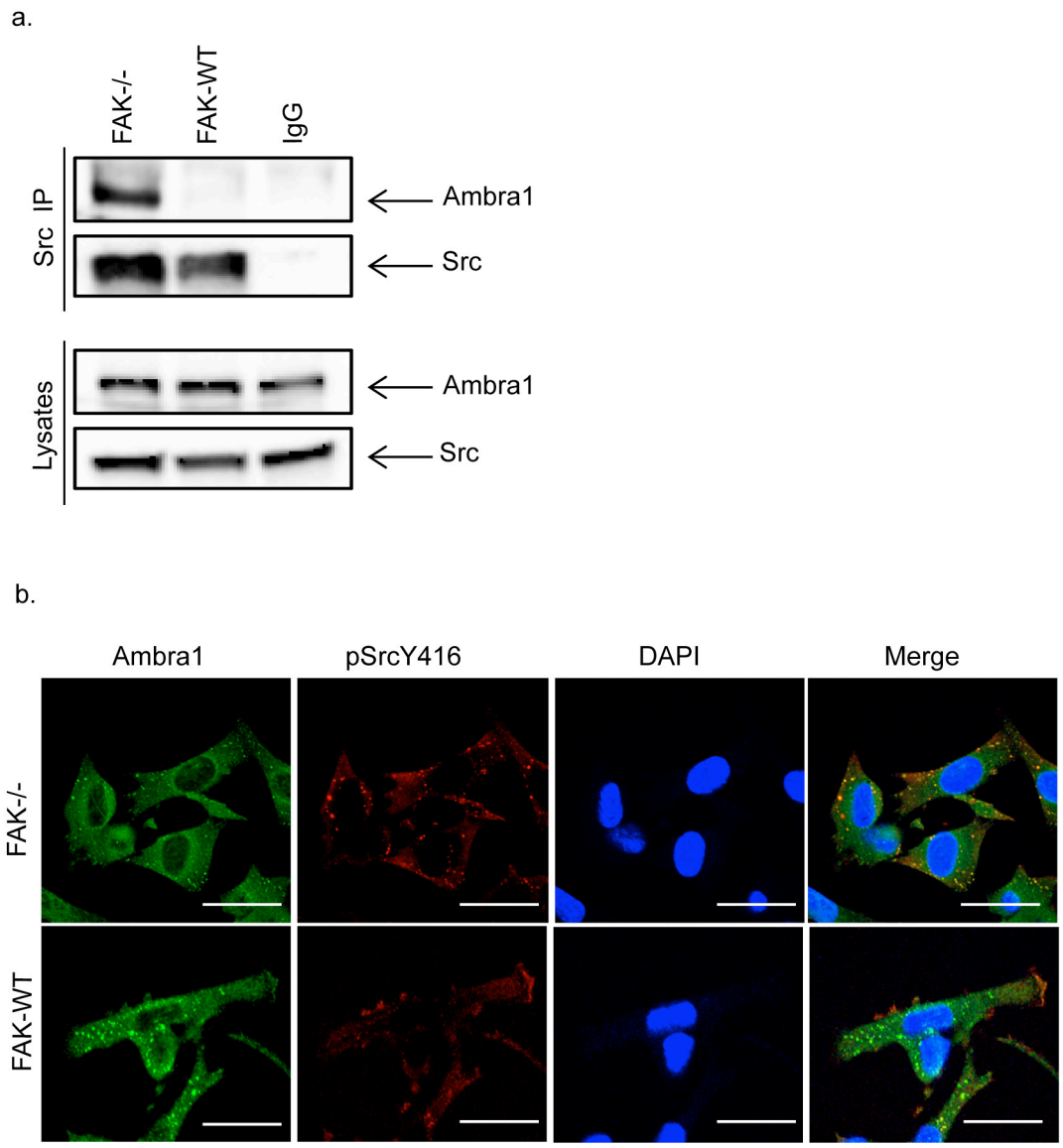
5.12 Ambra1 phosphorylation is affected in FAK-/- SCC cells

FAK-/- and FAK-WT SCC cell lysates were immunoprecipitated with anti-phosho-Tyrosine (pTyr20) antibody and then immunoblotted with anti-Ambra1 antibody. Cell lysates were also probed with anti-Ambra1 antibody. IgG control was also included.

First we investigated whether Ambra1 forms a complex with Src, and if so whether this was regulated by FAK. FAK-/- and FAK-WT SCC cell lysates were immunoprecipitated with anti-Src antibody and then immunoblotted with either anti-Ambra1 or anti-Src antibodies (Figure 5.13a). We found that there is not a detectable interaction between Src and Ambra1 in FAK-WT SCC cells. However there is a strong association between these two proteins in FAK-/- SCC cells.

Previously have shown that active Src co-localises with known autophagy regulators such as Atg12 and Atg5 (Sandilands et al., 2012a), and siRNA of these proteins can inhibit Src targeting to the autophagosomes. We investigated whether there was co-localisation between p-Src-Y416 and Ambra1 in FAK-/- and FAK-WT cells. The cells were fixed and stained for Ambra1, p-Src-Y416 and DAPI (Figure 5.13b). This showed that some Ambra1 co-localise with p-Src-Y416 at focal adhesions in FAK-WT cells, and in puncta that were previously identified as autophagosomes in the cytoplasm of FAK-/- SCC cells.

These data imply that Ambra1 forms a complex with Src and co-localises with p-Src-Y416 in autophagic puncta of FAK-/- cells, but not obviously in FAK-WT SCC cells.



5.13 Ambra1 associates with Src and co-localise with active Src in FAK-/- cells

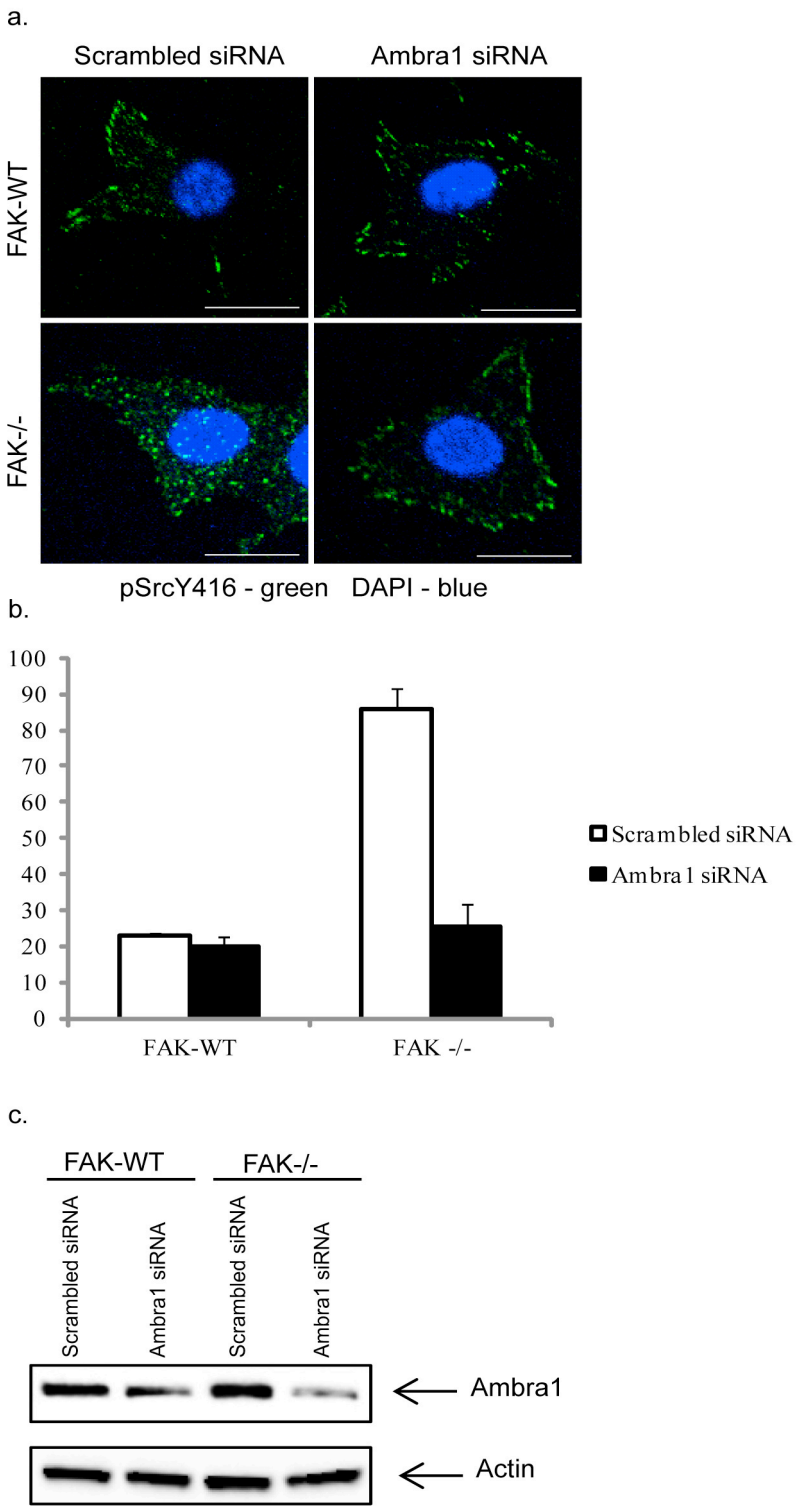
(a) Cell lysates from FAK-/- or FAK-WT SCC cells were immunoprecipitated with anti-Src antibody. Purified complexes and corresponding total cell lysates were analysed with SDS-PAGE and immunoblotted using anti-Ambra1 and anti-Src antibodies. IgG control is also included (b) FAK-/- (upper panel) and FAK-WT (lower panel) SCC cells were fixed and stained with anti-pSrcY416 (red), anti-Ambra1 (green) and DAPI (blue). Merged images are also shown. Scale bar 20um

5.1.12 Ambra1 siRNA in SCC cells

Next we investigated the effect of Ambra1 knockdown on localization of active Src in SCC cells. FAK-/- and FAK-WT SCC cells were transfected with either scrambled or Ambra1 siRNA (100nM). After 24 hours the cells were plated on coverslips and left for 24 hours before they were fixed and stained for p-Src-Y416 and DAPI (Figure 5.14a). Cells with Src in intracellular puncta were counted and quantified (Figure 5.14b). Scrambled or Ambra1 siRNA transfected SCC cells were also lysed and cell lysates were examined for Ambra1 knockdown by immunoblotting using anti-Ambra1 or anti-actin antibodies (Figure 5.14c). We found that Ambra1 knockdown has no effect on focal adhesion localisation of active Src in FAK-WT SCC cells; however active Src localisation to intracellular puncta was inhibited and active Src was restored to the cell periphery when FAK-/- SCC cells were transfected with Ambra1 siRNA. Thus, Ambra1 is a key mediator of the trafficking of active Src to autophagosomes when FAK is absent. Sine Ambra1 is involved in the formation of autophagosomes, we assume that its role is also in the formation of structures required to generate the Src-containing autophagic puncta in FAK-deficient cancer cells. It binds to FAK, and upon FAK loss, Ambra1 forms a complex with active Src and co-traffics to autophagic puncta in a manner that depends on Ambra1.

Due to time constraints, I was unable to determine the role of Ambra1 in polarisation and invasion of the SCC cells in vitro. However, this work is on-going in the Frame lab at the moment. We are interested to know whether there is a general link between autophagy regulators, in controlling steady state levels of adhesion signalling proteins like FAK and Src, especially upon adhesion stress (such as when FAK is deleted) and polarisation and invasion that are regulated by the same proteins.

Moreover, SCC cancer cells use autophagy to deal with high levels of active tyrosine kinases (TKs), like Src and Ret, when their scaffolding protein FAK is missing. Therefore understanding this pathway may reveal important new targets that prevent survival of cancer cells via autophagy when cells are under adhesion stress. In this work, we identified Ambra1 as a new mediator of Src-selective autophagy. However, Ambra1 is not an enzyme and not quite clear how it might be targeted since generating of small molecular inhibitors of protein-protein interactions (PPIs) is hard. The major challenge to target PPIs is that protein-protein binding involves large surface areas, which are difficult to disrupt using small compounds. In addition, many protein-protein contact surfaces lack defined pockets suitable for small molecules to bind. Another challenge is that in some cases, the protein domain involved in PPIs may be unfolded until the confirmation is stabilised through binding to the interacting partner, which makes protein surface very difficult for the design of small molecular targets by using computational techniques [reviewed in (Jin et al., 2014; Zinzalla and Thurston, 2009)].



5.14 Ambra1 knockdown restores localisation of active Src to the cell periphery

FAK-/- and FAK-WT SCC cells transfected with either Scrambled or Ambra1 siRNA were (a) fixed and stained with anti-p-Src-Y416 (green) and DAPI (blue). Scale bars 20µm, (b) quantification of cells with Src in intracellular puncta, or (c) immunoblotted using anti-Ambra1 and anti-actin antibodies.

5.2 Discussion

Using phage display, peptide array analysis and co-immunoprecipitation experiments in SCC cells, we identified a potential between FAK and the autophagy regulatory protein Ambra1.

To validate this interaction, we utilised a polyclonal anti-Ambra1 antibody, manufactured by Sigma, raised against a 15 amino acid peptide from the C-terminus of human Ambra1. This antibody is supposed to specifically detect Ambra1 protein in human, rat and mouse and has been shown by Sigma to be suitable for use in immunoblotting. In our immunoblotting experiment using SCC cell lysates we consistently detected multiple species of different molecular weights between 80-120kDa. While full length Ambra1 is typically 140kDa, there are at least 7 different isoforms of Ambra1 protein, produced by alternative splicing, detected in mice. These vary in size between 83 kDa to 140 kDa (reference). Therefore, we concluded that the different bands observed in our immunoblots were likely different isoforms of Ambra1. We also found that these immune-reactive protein species were reduced in the absence of FAK in co-IP experiments. This antibody was not suitable for immunofluorescence as there was no staining, so we used an anti-Ambra1 antibody from Abcam that according to the manufacturer was suitable for this application. With this antibody we detected strong staining throughout the cell cytoplasm, a proportion of which seemed to co-localise with active Src in cytoplasmic puncta but not with FAK in focal adhesions.

After repeated failed attempts to knockdown Ambra1 in SCC cells using siRNA or shRNA, we obtained Ambra1-/- MEFs and their WT counterparts from the Velasco

and Cecconi groups. Using these cells, we observed the same banding pattern in our immunoblots (Sigma antibody) and the same intracellular localisation using immunofluoresence (Abcam antibody) in cells that were Ambra1+/+ and Ambra1-/- (Figure 5.6). These experiments confirmed that these commercial antibodies supposedly specific for Ambra1 were not, in fact, detecting Ambra1 but were either non-specific or were detecting other proteins within the molecular weight range reported for Ambra1 isoforms. This was very frustrating, and wasted significant amount of time, but led us to search more widely, and more carefully, for a specific bone fide Ambra1 antibody.

We tested a new anti-Ambra1 antibody from Millipore. We found that this antibody reacted specifically with a protein species ~150kDa, and antibody specificity was also confirmed by using Ambra1 siRNA and Ambra1-deficient MEFs. Therefore, we decided all experiments would now be repeated using this antibody.

Data presented in this chapter show that there is a physical interaction between FAK and Ambra1 proteins in SCC cells, which was demonstrated with co-IP experiments. However to show that this interaction is direct, *in vitro* binding assay needs to be done using recombinant Ambra1 and recombinant FAK proteins. While mapping potential binding sites suggests that there is a direct interaction between FAK and Ambra1 we will, in the future, make a binding effector mutant that will help to show the loss of FAK/Ambra1 interaction, and the effects of interfering in binding. However, the proline rich region within FAK that may interact with Ambra1 is also a potential binding site for p130Cas (Polte and Hanks, 1995), although different proline residues are involved.

Here we investigated how FAK/Ambra1 interaction was regulated and we found that FAK phosphorylation or its kinase activity was required for this interaction.

It has been reported that Ambra1 regulates autophagy as part of the Beclin1/PI3KC3 multi-protein complex and that loss of Ambra1 disrupts the Beclin1/PI3KC3 interaction, and therefore affects PI3KC3 activity (Fimia et al., 2007). We found that FAK also present in the Beclin1/PI3KC3 complex.

One of the mechanisms of autophagy regulation is altering the assembly of Beclin1/PI3KC3 core complex, which could affect PI3KC3 kinase activity. Several proteins have been identified that function as positive or negative regulators of autophagy. For example, UVRAG binds to Beclin1 and enhances Beclin1/PI3KC3 interaction and PI3KC3 kinase activity (Liang et al., 2006). Moreover, it has been shown that Bcl-2 binds to Beclin1 and inhibits the interaction between Beclin1 and PI3KC3, and therefore inhibits PI3KC3 activity (Pattingre et al., 2005). We examined endogenous Beclin1/PI3KC3 and PI3KC3/p150 interactions in SCC cells in the presence and absence of FAK. We found that FAK does not affect these interactions, implying that FAK is not required for Beclin1/PI3KC3 multi-protein complex formation.

Post-translational modification of autophagy proteins provides another mechanism of how autophagy is regulated. For example, mammalian Ste20-like kinase 1 (Mst1) which is a Serine/Threonine kinase, phosphorylates Beclin1 and inhibits autophagy by promoting the interaction between Beclin1 and Bcl-2 (Maejima et al., 2013). Moreover, Akt inhibits autophagy through phosphorylation of Beclin1 (Wang et al., 2012). Several studies have previously suggested that Ambra1 activity could be

modulated trough its phosphorylation. Autophagy induction leads ULK1-dependent phosphorylation of Ambra1, which in turn directs dissociation of Ambra1-DLC1 complex from the dynein complex, and re-localisation of Ambra1/Beclin1 complex to the ER, where autophagy is initiated (Di Bartolomeo et al., 2010). The latter study shows that ULK1 activates Ambra1 by phosphorylation. However, phosphorylation of Ambra1 could have a negative regulatory effect on autophagy, for example, mTOR could inhibit ULK1 stability and activity by phosphorylation of Ambra1 at Ser52 (Nazio et al., 2013). To examine the potential role of FAK in the process of Ambra1 phosphorylation, we performed immunoprecipitation experiment in FAK-/- and FAK-WT SCC cells using anti-Tyr antibody. Phosphorylation of Ambra1 on tyrosine residues has never been shown before. Our data suggest that FAK binds to Ambra1 and either directly phosphorylates it or acts as a scaffold for other protein tyrosine kinase. FAK mediated Ambra1 phosphorylation may negatively regulates autophagy by disrupting the interaction between Ambra1 and E3-ligase TRAF6, which has been shown to support ULK1 ubiquitylation, and its subsequent stabilisation and function (Nazio et al., 2013). When FAK is absent, Ambra1 is dephosphorylated. In this condition, Ambra1 may be able to interact with TRAF6 and enhance ULK1 kinase activity and stability, which in turn leads to ULK1-dependent Ambra1 phosphorylation and activation.

Although we don't know if FAK/Ambra1 interaction plays a role in selective autophagy of active Src in SCC cells, we have shown that Ambra1 itself is important in this process. When we knockdown Ambra1 expression in FAK -/- SCC cells and examine localisation of active Src, we found that p-Src-Y416 localisation was restored to focal adhesions in FAK-/- cells. This is in line with the effect of inhibition

of several Atg proteins, such as the autophagy regulatory Atg5 and Atg12, whose inhibition promotes localisation of Src to focal adhesions (Sandilands et al., 2012a). Interestingly, we also saw co-localisation and binding between Src and Ambra1, suggesting that Ambra1 may play an additional role than just regulation of general autophagy. Since Ambra1 is in a complex with active Src when FAK is absent, and is required for Src targeting to autophagosomes, Ambra1 may be required for the formation of these autophagosomes at focal adhesions.

Most of the previous studies on Ambra1 were carried out in relation to its roles in neurobiology and starvation-induced autophagy, but the role of Ambra1 in cancer biology still remains to be elucidated. Some studies have suggested that autophagy is important in cancer cell survival (Buchser et al., 2012; Sandilands et al., 2012b; Schoenlein et al., 2009), such as autophagy promotes SCC cell survival by modulating the activity of Src, and a recent paper has demonstrated that Ambra1 promotes autophagy and regulates tumour cell survival in colorectal cancer cells (Gu et al., 2014). In addition, it has been reported that Ambra1 overexpression is associated with poor outcome in patients with pancreatic ductal adenocarcinoma, such as Ambra1 expression positively correlates with the expression of Snail, and promotes hepatocellular carcinoma cell invasion through the induction of EMT (Nitta et al., 2014). Therefore we think that further studies of FAK/Ambra1 interaction could contribute to better understanding of the role of Ambra1 in processes involved in cancer. A wider study looking at Ambra1 in SCC and other cancers would be interesting.

Summary

The main findings of this study are:

1. We identified Ambra1 as a novel FAK binding protein
2. It appears that FAK phosphorylation or its kinase activity is required for optimal FAK/Ambra1 interaction
3. FAK not only binds to Ambra1, but it is also a part of the Beclin1/PI3KC3 multi-protein core complex
4. FAK is not required for Beclin1/PI3KC3 multi-protein complex formation
5. FAK directly or indirectly regulates tyrosine phosphorylation of Ambra1 – although we do not yet know the significance of this
6. We found that Ambra1 associates with Src specifically in FAK-/- SCC cells, and co-localise in intracellular puncta
7. Ambra1 is involved in the trafficking of active Src to autophagic puncta in absence of FAK, and most likely in the formation of these structures

Future work

Characterisation roles of Ambra1 in cancer cells

1. There is a possibility that there is a general link between autophagy regulators, in controlling the steady state levels of adhesion signaling molecules such as Src and FAK, and cancer associated cellular processes that are mediated by the same proteins such as cell polarisation, migration and invasion. Therefore it would be interesting to examine the effects of Ambra1 siRNA on SCC cell migration, proliferation, polarisation and invasion
2. To identify the molecular mechanisms of Ambra1 function, Use Reverse Phase Protein Array (RPPA) technology to establish differences in protein expression in SCC cells expressing Ambra1 siRNA or in Ambra-/- and

Ambra1+/+ MEFs under different conditions i.e. in suspension (adhesion stress) or upon nutrient deprivation

3. Use mass spectrometry to identify Ambra1 binding partners in the presence and absence of FAK which help us understand the mechanism how SCC cells use autophagy to deal with high levels of TKs, such as Src and Ret

Characterisation of FAK/Ambra1 interaction

1. Make an effector mutant FAK protein that can't bind to Ambra1
2. Make stable cell line expressing effector mutant
3. Validate binding mutant: a) confirm the loss of interaction with Ambra1 b) confirm that mutations do not affect FAK binding with other known interacting partners, such as p130Cas
4. Characterise any changes that loss of FAK/Ambra1 interaction makes, firstly look at the localisation of active Src
5. Use FAK-WT and FAK-/- cells, and FAK-/- cells re-expressing different FAK phosphorylation mutants, and also FAK and Src inhibitors to further investigate the role of FAK in Ambra1 phosphorylation
6. Look at the interaction between Ambra1 and the TRAF6 ubiquitin ligase in the absence and presence of FAK
7. Analyse the protein level and Lys-63-linked ubiquitylation of ULK1 in FAK-/- and FAK-WT cells
8. Look at the processes that FAK known to play a role in, such as cell migration, invasion, cell polarity and cell spreading

I have found two new binding partners important for different aspects of FAK biology – Axl that is involved in the regulation of SCC cell invasion, and Ambra1 that is involved in the trafficking of active Src in autophagic puncta when FAK is absent - each may be important scaffolding functions mediated by FAK.

Chapter 6

Summary and Perspectives

6 Summary and Perspectives

FAK is a non-receptor protein tyrosine kinase that plays an important role in signal transduction from integrins and growth factor receptors and implicated in many cellular processes including cell proliferations, cell survival, cell migration and spreading. FAK is involved in the development and progression of many cancer types. However, how FAK regulates these processes needs to be elucidated, including whether or not it works predominantly via substrate phosphorylation or by its adaptor protein-protein interaction functions that permit the tethering of proteins into complexes at integrin adhesions, and potentially also at other sub-cellular locations. The purpose of the work presented in this thesis was to identify new interacting partners of FAK, and to address their roles in cancer-associated cell biology processes, determining whether FAK and its partner binding proteins have key roles. If this is the case, then disruption of these interactions in cells, in which FAK-mediated processes are mis-regulated, could be useful for therapeutic purposes for cancer treatment. However, it is well known that targeting protein-protein interactions is not a popular choice of therapeutic strategy – due to the difficulties in generating specific and high affinity inhibitors. Therefore, it may be valuable to identify new targets, by examining partners of key proteins like FAK, which are more easily targeted by small molecular inhibitors.

By using *in vitro* screening methods, namely protein microarray and phage display, which were already established in our laboratory, I was able to identify, and validate, novel FAK interactions. These techniques provide quick and simple strategies for examination of protein-protein interactions on a large scale. However, the

disadvantages of using these in vitro techniques are that both protein microarray and phage display lack posttranslational protein modifications, which are often important for many protein functions. Therefore, using mass spectrometry for identification of novel protein-protein interactions would be ideal, and is now the method of choice in on-going studies.

To characterise my novel FAK interactions in vitro, I used an SCC cancer cell model. This was an excellent model, because we could delete the gene encoding FAK and reconstitute with WT and mutant FAK proteins. This provided a genetically 'clean' system in which to study the role of FAK, or its partner proteins, in aspects of cell biology. FAK-/- SCC cells re-expressing various FAK mutants enabled me to analyse the contribution of individual phosphorylation sites and domains of FAK in specific interactions.

Identification of Axl as FAK interacting protein

Through combinations of immunoprecipitation experiments with *in vitro* binding and pulldown assays, I confirmed the direct interactions between FAK and Axl proteins. Moreover, I demonstrated that FAK/Axl interaction is not regulated by any of the major phosphorylation events characterised for FAK or by its kinase activity. We therefore hypothesised that the function of FAK in this regard is as a largely un-regulated scaffold protein, and Axl knockdown did not affect FAK phosphorylation.

Axl itself is overexpressed in a variety of cancers and plays some role in tumour growth and metastasis in a number of contexts. Moreover, Axl over-expression often associates with poor prognosis [reviewed in (Paccez et al., 2014)]. I found that the predominant role of Axl in SCC cells was to control cell spreading, cell polarisation

and invasive migration in vitro, while cell proliferation was independent of Axl. However, we don't know whether Axl regulates these invasion-associated processes through its interaction with FAK – although these processes also require FAK as published by the Frame lab, and others, previously. Therefore, while further study is required to analyse the importance of this interaction in SCC cell biology, it may be that Axl is a potentially useful anti-invasion target. There are Axl inhibitors in pre-clinical and clinical development, and it will be interesting to assess their roles in SCC cancer phenotypes *in vitro* and *in vivo*.

Identification of Ambra1 as FAK interacting protein

Previous studies in our laboratory demonstrated that in FAK-/- SCC cells, active Src is selected for degradation through the autophagy pathway, and this is an adaptive mechanism to permit SCC cell survival upon adhesion stress. When FAK-WT is re-expressed in FAK-/- cells, localisation of active Src is restored to focal adhesions, suggesting a negative regulatory role of FAK in selective autophagy of active Src (Sandilands et al., 2012a). This work prompted us to also study Ambra1, reported to be an autophagy regulatory protein that binds to Beclin1, promoting its interaction with PI3KC3, and positively regulating the early stages of autophagy (Fimia et al., 2007).

In this thesis, I identified an interaction between FAK and Ambra1, validated in SCC cancer cells. I report that this interaction requires FAK phosphorylation and kinase activity. Peptide array analysis and alanine scanning showed that FAK likely interacts with Ambra1 through a C-terminal proline rich region, which was previously identified as close to a binding site for p130Cas protein; however

different proline residues were involved in the latter interaction. Ongoing work in the Frame lab is generating effector binding mutant proteins predicted to impair the binding of Ambra1 to FAK, with a view to re-expressing this in FAK-deficient cells and analysing whether cancer-associated functions are impaired. Thus far, we know that Ambra1 complexes with FAK and is tyrosine phosphorylated, but when FAK is absent after genetic deletion, Ambra1 forms a complex with active Src. This association of Src with Ambra1 might bring it into complex with the Beclin1/PI3KC3 and promote PI3KC3 activity in the vicinity of adhesion sites, so triggering formation of autophagosomes that ultimately remove excess, highly active Src from focal adhesions, and for degradation [as described in (Sandilands et al., 2012b)]. In keeping with this, I have shown that Ambra1 knockdown inhibits targeting of active Src to autophagosomes initiated at focal adhesions.

This work raises the question of what role the FAK/Ambra1 interaction plays when FAK is present at high levels, as in the SCC model cells we used here and in many cancer cells. To speculate, it is possible that Ambra1 also regulates FAK-mediated, cancer associated cellular processes like polarisation and invasive migration. If true, this would raise the exciting possibility that autophagy proteins are playing a dual role - in autophagy on the one hand and on invasion on the other – dependent on which specific cellular protein complexes form. It may be that autophagy and invasion are linked and that autophagy is performing a key function to tightly control the levels of highly active kinases at adhesion sites and permit their invasive regulatory functions. This remains to be tested, and work is ongoing to determine the role of Ambra1, and the FAK-Ambra1 complex, in a full range of cancer phenotypes in vitro and in vivo.

Abbi, S. and Guan, J. L. (2002). Focal adhesion kinase: protein interactions and cellular functions. *Histol Histopathol* **17**, 1163-71.

Abbi, S., Ueda, H., Zheng, C., Cooper, L. A., Zhao, J., Christopher, R. and Guan, J. L. (2002). Regulation of focal adhesion kinase by a novel protein inhibitor FIP200. *Mol Biol Cell* **13**, 3178-91.

Abedi, H. and Zachary, I. (1995). Signalling mechanisms in the regulation of vascular cell migration. *Cardiovasc Res* **30**, 544-56.

Abu-Ghazaleh, R., Kabir, J., Jia, H., Lobo, M. and Zachary, I. (2001). Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells. *Biochem J* **360**, 255-64.

Agochiya, M., Brunton, V. G., Owens, D. W., Parkinson, E. K., Paraskeva, C., Keith, W. N. and Frame, M. C. (1999). Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells. *Oncogene* **18**, 5646-53.

Angenendt, P. (2005). Progress in protein and antibody microarray technology. *Drug Discov Today* **10**, 503-11.

Arias-Romero, L. E., Saha, S., Villamar-Cruz, O., Yip, S. C., Ethier, S. P., Zhang, Z. Y. and Chernoff, J. (2009). Activation of Src by protein tyrosine phosphatase 1B Is required for ErbB2 transformation of human breast epithelial cells. *Cancer Res* **69**, 4582-8.

Avraham, H., Park, S. Y., Schinkmann, K. and Avraham, S. (2000). RAFTK/Pyk2-mediated cellular signalling. *Cell Signal* **12**, 123-33.

Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Pasztor, L. M., White, R. A., Groopman, J. E. et al. (1995). Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *J Biol Chem* **270**, 27742-51.

Balla, T. (2005). Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. *J Cell Sci* **118**, 2093-104.

Barberis, L., Wary, K. K., Fiucci, G., Liu, F., Hirsch, E., Brancaccio, M., Altruda, F., Tarone, G. and Giancotti, F. G. (2000). Distinct roles of the adaptor protein Shc and focal adhesion kinase in integrin signaling to ERK. *J Biol Chem* **275**, 36532-40.

Bellas, R. E., Harrington, E. O., Sheahan, K. L., Newton, J., Marcus, C. and Rounds, S. (2002). FAK blunts adenosine-homocysteine-induced endothelial cell apoptosis: requirement for PI 3-kinase. *Am J Physiol Lung Cell Mol Physiol* **282**, L1135-42.

Benlimame, N., He, Q., Jie, S., Xiao, D., Xu, Y. J., Loignon, M., Schlaepfer, D. D. and Alaoui-Jamali, M. A. (2005). FAK signaling is critical for

ErbB-2/ErbB-3 receptor cooperation for oncogenic transformation and invasion. *J Cell Biol* **171**, 505-16.

Bergers, G. and Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* **3**, 401-10.

Bertone, P. and Snyder, M. (2005). Advances in functional protein microarray technology. *FEBS J* **272**, 5400-11.

Bjorge, J. D., Jakymiw, A. and Fujita, D. J. (2000). Selected glimpses into the activation and function of Src kinase. *Oncogene* **19**, 5620-35.

Block, M. R., Badowski, C., Millon-Fremillon, A., Bouvard, D., Bouin, A. P., Faurobert, E., Gerber-Scokaert, D., Planus, E. and Albiges-Rizo, C. (2008). Podosome-type adhesions and focal adhesions, so alike yet so different. *Eur J Cell Biol* **87**, 491-506.

Boggon, T. J. and Eck, M. J. (2004). Structure and regulation of Src family kinases. *Oncogene* **23**, 7918-27.

Bond, M., Sala-Newby, G. B. and Newby, A. C. (2004). Focal adhesion kinase (FAK)-dependent regulation of S-phase kinase-associated protein-2 (Skp-2) stability. A novel mechanism regulating smooth muscle cell proliferation. *J Biol Chem* **279**, 37304-10.

Bose, R., Kavuri, S. M., Searleman, A. C., Shen, W., Shen, D., Koboldt, D. C., Monsey, J., Goel, N., Aronson, A. B., Li, S. et al. (2013). Activating HER2 mutations in HER2 gene amplification negative breast cancer. *Cancer Discov* **3**, 224-37.

Boya, P., Reggiori, F. and Codogno, P. (2013). Emerging regulation and functions of autophagy. *Nat Cell Biol* **15**, 713-20.

Broome, M. A. and Hunter, T. (1997). The PDGF receptor phosphorylates Tyr 138 in the c-Src SH3 domain in vivo reducing peptide ligand binding. *Oncogene* **14**, 17-34.

Brown, M. C., Cary, L. A., Jamieson, J. S., Cooper, J. A. and Turner, C. E. (2005). Src and FAK kinases cooperate to phosphorylate paxillin kinase linker, stimulate its focal adhesion localization, and regulate cell spreading and protrusiveness. *Mol Biol Cell* **16**, 4316-28.

Buchser, W. J., Laskow, T. C., Pavlik, P. J., Lin, H. M. and Lotze, M. T. (2012). Cell-mediated autophagy promotes cancer cell survival. *Cancer Res* **72**, 2970-9.

Budagian, V., Bulanova, E., Orinska, Z., Duitman, E., Brandt, K., Ludwig, A., Hartmann, D., Lemke, G., Saftig, P. and Bulfone-Paus, S. (2005). Soluble Axl is generated by ADAM10-dependent cleavage and associates with Gas6 in mouse serum. *Mol Cell Biol* **25**, 9324-39.

Burgaya, F. and Girault, J. A. (1996). Cloning of focal adhesion kinase, pp125FAK, from rat brain reveals multiple transcripts with different patterns of expression. *Brain Res Mol Brain Res* **37**, 63-73.

Burgaya, F., Toutant, M., Studler, J. M., Costa, A., Le Bert, M., Gelman, M. and Girault, J. A. (1997). Alternatively spliced focal adhesion kinase in rat brain with increased autophosphorylation activity. *J Biol Chem* **272**, 28720-5.

Burridge, K. and Connell, L. (1983). A new protein of adhesion plaques and ruffling membranes. *J Cell Biol* **97**, 359-67.

Cai, X., Lietha, D., Ceccarelli, D. F., Karginov, A. V., Rajfur, Z., Jacobson, K., Hahn, K. M., Eck, M. J. and Schaller, M. D. (2008). Spatial and temporal regulation of focal adhesion kinase activity in living cells. *Mol Cell Biol* **28**, 201-14.

Calalb, M. B., Polte, T. R. and Hanks, S. K. (1995). Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol Cell Biol* **15**, 954-63.

Campellone, K. G. and Welch, M. D. (2010). A nucleator arms race: cellular control of actin assembly. *Nat Rev Mol Cell Biol* **11**, 237-51.

Canel, M., Secades, P., Garzon-Arango, M., Allonca, E., Suarez, C., Serrels, A., Frame, M., Brunton, V. and Chiara, M. D. (2008). Involvement of focal adhesion kinase in cellular invasion of head and neck squamous cell carcinomas via regulation of MMP-2 expression. *Br J Cancer* **98**, 1274-84.

Carragher, N. O., Walker, S. M., Scott Carragher, L. A., Harris, F., Sawyer, T. K., Brunton, V. G., Ozanne, B. W. and Frame, M. C. (2006). Calpain 2 and Src dependence distinguishes mesenchymal and amoeboid modes of tumour cell invasion: a link to integrin function. *Oncogene* **25**, 5726-40.

Carter, N., Nakamoto, T., Hirai, H. and Hunter, T. (2002). EphrinA1-induced cytoskeletal re-organization requires FAK and p130(cas). *Nat Cell Biol* **4**, 565-73.

Cary, L. A., Chang, J. F. and Guan, J. L. (1996). Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *J Cell Sci* **109 (Pt 7)**, 1787-94.

Casanovas, O., Hicklin, D. J., Bergers, G. and Hanahan, D. (2005). Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* **8**, 299-309.

Ceccarelli, D. F., Song, H. K., Poy, F., Schaller, M. D. and Eck, M. J. (2006). Crystal structure of the FERM domain of focal adhesion kinase. *J Biol Chem* **281**, 252-9.

Chan, C. M., Jing, X., Pike, L. A., Zhou, Q., Lim, D. J., Sams, S. B., Lund, G. S., Sharma, V., Haugen, B. R. and Schweppe, R. E. (2012). Targeted inhibition of Src kinase with dasatinib blocks thyroid cancer growth and metastasis. *Clin Cancer Res* **18**, 3580-91.

Chan, P. C., Lai, J. F., Cheng, C. H., Tang, M. J., Chiu, C. C. and Chen, H. C. (1999). Suppression of ultraviolet irradiation-induced apoptosis by overexpression of focal adhesion kinase in Madin-Darby canine kidney cells. *J Biol Chem* **274**, 26901-6.

Chen, H. C., Appeddu, P. A., Isoda, H. and Guan, J. L. (1996). Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J Biol Chem* **271**, 26329-34.

Chen, H. C., Appeddu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D. and Guan, J. L. (1995). Interaction of focal adhesion kinase with cytoskeletal protein talin. *J Biol Chem* **270**, 16995-9.

Chen, H. J., Lin, C. M., Lin, C. S., Perez-Olle, R., Leung, C. L. and Liem, R. K. (2006). The role of microtubule actin cross-linking factor 1 (MACF1) in the Wnt signaling pathway. *Genes Dev* **20**, 1933-45.

Chen, J., Elfiky, A., Han, M., Chen, C. and Saif, M. W. (2014). The role of Src in colon cancer and its therapeutic implications. *Clin Colorectal Cancer* **13**, 5-13.

Chen, J. S., Huang, X. H., Wang, Q., Huang, J. Q., Zhang, L. J., Chen, X. L., Lei, J. and Cheng, Z. X. (2013). Sonic hedgehog signaling pathway induces cell migration and invasion through focal adhesion kinase/AKT signaling-mediated activation of matrix metalloproteinase (MMP)-2 and MMP-9 in liver cancer. *Carcinogenesis* **34**, 10-9.

Chen, L. M., Bailey, D. and Fernandez-Valle, C. (2000). Association of beta 1 integrin with focal adhesion kinase and paxillin in differentiating Schwann cells. *J Neurosci* **20**, 3776-84.

Chen, R., Kim, O., Li, M., Xiong, X., Guan, J. L., Kung, H. J., Chen, H., Shimizu, Y. and Qiu, Y. (2001). Regulation of the PH-domain-containing tyrosine kinase Etk by focal adhesion kinase through the FERM domain. *Nat Cell Biol* **3**, 439-44.

Chen, S. Y. and Chen, H. C. (2006). Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. *Mol Cell Biol* **26**, 5155-67.

Chung, B. I., Malkowicz, S. B., Nguyen, T. B., Libertino, J. A. and McGarvey, T. W. (2003). Expression of the proto-oncogene Axl in renal cell carcinoma. *DNA Cell Biol* **22**, 533-40.

Cichon, M. A., Szentpetery, Z., Caley, M. P., Papadakis, E. S., Mackenzie, I. C., Brennan, C. H. and O'Toole, E. A. (2014). The receptor tyrosine kinase Axl regulates cell-cell adhesion and stemness in cutaneous squamous cell carcinoma. *Oncogene* **33**, 4185-92.

Cirri, P., Chiarugi, P., Marra, F., Raugei, G., Camici, G., Manao, G. and Ramponi, G. (1997). c-Src activates both STAT1 and STAT3 in PDGF-stimulated NIH3T3 cells. *Biochem Biophys Res Commun* **239**, 493-7.

Cohen, L. A. and Guan, J. L. (2005). Residues within the first subdomain of the FERM-like domain in focal adhesion kinase are important in its regulation. *J Biol Chem* **280**, 8197-207.

Cooper, L. A., Shen, T. L. and Guan, J. L. (2003). Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. *Mol Cell Biol* **23**, 8030-41.

Das, M., Subbayya Ithychanda, S., Qin, J. and Plow, E. F. (2014). Mechanisms of talin-dependent integrin signaling and crosstalk. *Biochim Biophys Acta* **1838**, 579-88.

De Luca, A., D'Alessio, A., Gallo, M., Maiello, M. R., Bode, A. M. and Normanno, N. (2014). Src and CXCR4 are involved in the invasiveness of breast cancer cells with acquired resistance to lapatinib. *Cell Cycle* **13**, 148-56.

Di Bartolomeo, S., Corazzari, M., Nazio, F., Oliverio, S., Lisi, G., Antonioli, M., Pagliarini, V., Matteoni, S., Fuoco, C., Giunta, L. et al. (2010). The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. *J Cell Biol* **191**, 155-68.

Dikic, I. and Schlessinger, J. (1998). Identification of a new Pyk2 isoform implicated in chemokine and antigen receptor signaling. *J Biol Chem* **273**, 14301-8.

Duncan, J. S. and Litchfield, D. W. (2008). Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. *Biochim Biophys Acta* **1784**, 33-47.

Dunn, K. B., Heffler, M. and Golubovskaya, V. M. (2010). Evolving therapies and FAK inhibitors for the treatment of cancer. *Anticancer Agents Med Chem* **10**, 722-34.

Dunty, J. M., Gabarra-Niecko, V., King, M. L., Ceccarelli, D. F., Eck, M. J. and Schaller, M. D. (2004). FERM domain interaction promotes FAK signaling. *Mol Cell Biol* **24**, 5353-68.

Egeblad, M. and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* **2**, 161-74.

Eliceiri, B. P., Puente, X. S., Hood, J. D., Stupack, D. G., Schlaepfer, D. D., Huang, X. Z., Sheppard, D. and Cheresh, D. A. (2002). Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. *J Cell Biol* **157**, 149-60.

Engen, J. R., Wales, T. E., Hochrein, J. M., Meyn, M. A., 3rd, Banu Ozkan, S., Bahar, I. and Smithgall, T. E. (2008). Structure and dynamic regulation of Src-family kinases. *Cell Mol Life Sci* **65**, 3058-73.

Etienne-Manneville, S. and Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* **106**, 489-98.

Ezratty, E. J., Partridge, M. A. and Gundersen, G. G. (2005). Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat Cell Biol* **7**, 581-90.

Fan, H. and Guan, J. L. (2011). Compensatory function of Pyk2 protein in the promotion of focal adhesion kinase (FAK)-null mammary cancer stem cell tumorigenicity and metastatic activity. *J Biol Chem* **286**, 18573-82.

Fan, H., Zhao, X., Sun, S., Luo, M. and Guan, J. L. (2013). Function of focal adhesion kinase scaffolding to mediate endophilin A2 phosphorylation

promotes epithelial-mesenchymal transition and mammary cancer stem cell activities in vivo. *J Biol Chem* **288**, 3322-33.

Fang, X. Q., Liu, X. F., Yao, L., Chen, C. Q., Gu, Z. D., Ni, P. H., Zheng, X. M. and Fan, Q. S. (2014). Somatic mutational analysis of FAK in breast cancer: a novel gain-of-function mutation due to deletion of exon 33. *Biochem Biophys Res Commun* **443**, 363-9.

Fauvel, B. and Yasri, A. (2014). Antibodies directed against receptor tyrosine kinases: current and future strategies to fight cancer. *MAbs* **6**, 838-51.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D. and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci U S A* **93**, 10887-90.

Feng, Y., He, D., Yao, Z. and Klionsky, D. J. (2014). The machinery of macroautophagy. *Cell Res* **24**, 24-41.

Fiedorek, F. T., Jr. and Kay, E. S. (1995). Mapping of the focal adhesion kinase (Fadk) gene to mouse chromosome 15 and human chromosome 8. *Mamm Genome* **6**, 123-6.

Fimia, G. M., Stoykova, A., Romagnoli, A., Giunta, L., Di Bartolomeo, S., Nardacci, R., Corazzari, M., Fuoco, C., Ucar, A., Schwartz, P. et al. (2007). Ambra1 regulates autophagy and development of the nervous system. *Nature* **447**, 1121-5.

Fingleton, B. (2006). Matrix metalloproteinases: roles in cancer and metastasis. *Front Biosci* **11**, 479-91.

Flinder, L. I., Wierod, L., Rosseland, C. M., Huitfeldt, H. S. and Skarpen, E. (2013). FAK regulates Cdk2 in EGF-stimulated primary cultures of hepatocytes. *J Cell Physiol* **228**, 1304-13.

Frame, M. C. (2002). Src in cancer: deregulation and consequences for cell behaviour. *Biochim Biophys Acta* **1602**, 114-30.

Frame, M. C., Patel, H., Serrels, B., Lietha, D. and Eck, M. J. (2010). The FERM domain: organizing the structure and function of FAK. *Nat Rev Mol Cell Biol* **11**, 802-14.

Frisch, S. M., Vuori, K., Ruoslahti, E. and Chan-Hui, P. Y. (1996). Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* **134**, 793-9.

Funderburk, S. F., Wang, Q. J. and Yue, Z. (2010). The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond. *Trends Cell Biol* **20**, 355-62.

Furuya, N., Yu, J., Byfield, M., Pattingre, S. and Levine, B. (2005). The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. *Autophagy* **1**, 46-52.

Gabriel, B., zur Hausen, A., Stickeler, E., Dietz, C., Gitsch, G., Fischer, D. C., Bouda, J., Tempfer, C. and Hasenburg, A. (2006). Weak expression of focal adhesion kinase (pp125FAK) in patients with cervical cancer is associated with poor disease outcome. *Clin Cancer Res* **12**, 2476-83.

Garcia, E. L. and Mills, A. A. (2002). Getting around lethality with inducible Cre-mediated excision. *Semin Cell Dev Biol* **13**, 151-8.

Gardel, M. L., Schneider, I. C., Aratyn-Schaus, Y. and Waterman, C. M. (2010). Mechanical integration of actin and adhesion dynamics in cell migration. *Annu Rev Cell Dev Biol* **26**, 315-33.

Gilmore, A. P. and Romer, L. H. (1996). Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Mol Biol Cell* **7**, 1209-24.

Girault, J. A., Labesse, G., Mornon, J. P. and Callebaut, I. (1999). The N-termini of FAK and JAKs contain divergent band 4.1 domains. *Trends Biochem Sci* **24**, 54-7.

Gjerdrum, C., Tiron, C., Hoiby, T., Stefansson, I., Haugen, H., Sandal, T., Collett, K., Li, S., McCormack, E., Gjertsen, B. T. et al. (2010). Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proc Natl Acad Sci U S A* **107**, 1124-9.

Golubovskaya, V. M. (2014). Targeting FAK in human cancer: from finding to first clinical trials. *Front Biosci (Landmark Ed)* **19**, 687-706.

Golubovskaya, V. M., Finch, R. and Cance, W. G. (2005). Direct interaction of the N-terminal domain of focal adhesion kinase with the N-terminal transactivation domain of p53. *J Biol Chem* **280**, 25008-21.

Golubovskaya, V. M., Finch, R., Kweh, F., Massoll, N. A., Campbell-Thompson, M., Wallace, M. R. and Cance, W. G. (2008a). p53 regulates FAK expression in human tumor cells. *Mol Carcinog* **47**, 373-82.

Golubovskaya, V. M., Ho, B., Zheng, M., Magis, A., Ostrov, D., Morrison, C. and Cance, W. G. (2013a). Disruption of focal adhesion kinase and p53 interaction with small molecule compound R2 reactivated p53 and blocked tumor growth. *BMC Cancer* **13**, 342.

Golubovskaya, V. M., Palma, N. L., Zheng, M., Ho, B., Magis, A., Ostrov, D. and Cance, W. G. (2013b). A small-molecule inhibitor, 5'-O-tritylthymidine, targets FAK and Mdm-2 interaction, and blocks breast and colon tumorigenesis in vivo. *Anticancer Agents Med Chem* **13**, 532-45.

Golubovskaya, V. M., Virnig, C. and Cance, W. G. (2008b). TAE226-induced apoptosis in breast cancer cells with overexpressed Src or EGFR. *Mol Carcinog* **47**, 222-34.

Gonzalez-Ruiz, D. and Gohlke, H. (2006). Targeting protein-protein interactions with small molecules: challenges and perspectives for computational binding epitope detection and ligand finding. *Curr Med Chem* **13**, 2607-25.

Green, J., Ikram, M., Vyas, J., Patel, N., Proby, C. M., Ghali, L., Leigh, I. M., O'Toole, E. A. and Storey, A. (2006). Overexpression of the Axl tyrosine kinase receptor in cutaneous SCC-derived cell lines and tumours. *Br J Cancer* **94**, 1446-51.

Gu, W., Wan, D., Qian, Q., Yi, B., He, Z., Gu, Y., Wang, L. and He, S. (2014). Ambra1 is an essential regulator of autophagy and apoptosis in SW620 cells: pro-survival role of Ambra1. *PLoS One* **9**, e90151.

Guinamard, R., Okigaki, M., Schlessinger, J. and Ravetch, J. V. (2000). Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat Immunol* **1**, 31-6.

Guo, J., Meng, F., Fu, X., Song, B., Yan, X. and Zhang, G. (2004). N-methyl-D-aspartate receptor and L-type voltage-gated Ca2+ channel activation mediate proline-rich tyrosine kinase 2 phosphorylation during cerebral ischemia in rats. *Neurosci Lett* **355**, 177-80.

Hafizi, S. and Dahlback, B. (2006). Signalling and functional diversity within the Axl subfamily of receptor tyrosine kinases. *Cytokine Growth Factor Rev* **17**, 295-304.

Hagel, M., George, E. L., Kim, A., Tamimi, R., Opitz, S. L., Turner, C. E., Imamoto, A. and Thomas, S. M. (2002). The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. *Mol Cell Biol* **22**, 901-15.

Halder, J., Kamat, A. A., Landen, C. N., Jr., Han, L. Y., Lutgendorf, S. K., Lin, Y. G., Merritt, W. M., Jennings, N. B., Chavez-Reyes, A., Coleman, R. L. et al. (2006). Focal adhesion kinase targeting using in vivo short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. *Clin Cancer Res* **12**, 4916-24.

Halder, J., Lin, Y. G., Merritt, W. M., Spannuth, W. A., Nick, A. M., Honda, T., Kamat, A. A., Han, L. Y., Kim, T. J., Lu, C. et al. (2007). Therapeutic efficacy of a novel focal adhesion kinase inhibitor TAE226 in ovarian carcinoma. *Cancer Res* **67**, 10976-83.

Hall, D. A., Ptacek, J. and Snyder, M. (2007). Protein microarray technology. *Mech Ageing Dev* **128**, 161-7.

Hall, J. E., Fu, W. and Schaller, M. D. (2011). Focal adhesion kinase: exploring Fak structure to gain insight into function. *Int Rev Cell Mol Biol* **288**, 185-225.

Han, D. C. and Guan, J. L. (1999). Association of focal adhesion kinase with Grb7 and its role in cell migration. *J Biol Chem* **274**, 24425-30.

Han, D. C., Shen, T. L. and Guan, J. L. (2000). Role of Grb7 targeting to focal contacts and its phosphorylation by focal adhesion kinase in regulation of cell migration. *J Biol Chem* **275**, 28911-7.

Hanks, S. K., Calalb, M. B., Harper, M. C. and Patel, S. K. (1992). Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc Natl Acad Sci U S A* **89**, 8487-91.

Harris, K. F., Shoji, I., Cooper, E. M., Kumar, S., Oda, H. and Howley, P. M. (1999). Ubiquitin-mediated degradation of active Src tyrosine kinase. *Proc Natl Acad Sci U S A* **96**, 13738-43.

Hauck, C. R., Hsia, D. A., Ilic, D. and Schlaepfer, D. D. (2002). v-Src SH3-enhanced interaction with focal adhesion kinase at beta 1 integrin-containing invadopodia promotes cell invasion. *J Biol Chem* **277**, 12487-90.

He, C. and Klionsky, D. J. (2009). Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet* **43**, 67-93.

Heidkamp, M. C., Bayer, A. L., Kalina, J. A., Eble, D. M. and Samarel, A. M. (2002). GFP-FRNK disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes. *Circ Res* **90**, 1282-9.

Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1993). Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *J Cell Biol* **123**, 993-1005.

Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1995). Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxyl terminal domain of focal adhesion kinase. *Mol Biol Cell* **6**, 637-47.

Hildebrand, J. D., Taylor, J. M. and Parsons, J. T. (1996). An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase. *Mol Cell Biol* **16**, 3169-78.

Holland, S. J., Pan, A., Franci, C., Hu, Y., Chang, B., Li, W., Duan, M., Torneros, A., Yu, J., Heckrodt, T. J. et al. (2010). R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Res* **70**, 1544-54.

Hood, J. D. and Cheresh, D. A. (2002). Role of integrins in cell invasion and migration. *Nat Rev Cancer* **2**, 91-100.

Hotary, K., Allen, E., Punturieri, A., Yana, I. and Weiss, S. J. (2000). Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. *J Cell Biol* **149**, 1309-23.

Hsia, D. A., Mitra, S. K., Hauck, C. R., Streblow, D. N., Nelson, J. A., Ilic, D., Huang, S., Li, E., Nemerow, G. R., Leng, J. et al. (2003). Differential regulation of cell motility and invasion by FAK. *J Cell Biol* **160**, 753-67.

Huang, Y. J., Frazier, M. L., Zhang, N., Liu, Q. and Wei, C. (2014). Reverse-phase protein array analysis to identify biomarker proteins in human pancreatic cancer. *Dig Dis Sci* **59**, 968-75.

Hubbard, S. R. and Miller, W. T. (2007). Receptor tyrosine kinases: mechanisms of activation and signaling. *Curr Opin Cell Biol* **19**, 117-23.

Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G. and Otey, C. A. (1996). Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. *J Cell Biol* **135**, 1383-90.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377**, 539-44.

Infante, J. R., Camidge, D. R., Mileshkin, L. R., Chen, E. X., Hicks, R. J., Rischin, D., Fingert, H., Pierce, K. J., Xu, H., Roberts, W. G. et al. (2012). Safety, pharmacokinetic, and pharmacodynamic phase I dose-escalation trial of PF-00562271, an inhibitor of focal adhesion kinase, in advanced solid tumors. *J Clin Oncol* **30**, 1527-33.

Ingley, E. (2008). Src family kinases: regulation of their activities, levels and identification of new pathways. *Biochim Biophys Acta* **1784**, 56-65.

Irby, R. B., Mao, W., Coppola, D., Kang, J., Loubeau, J. M., Trudeau, W., Karl, R., Fujita, D. J., Jove, R. and Yeatman, T. J. (1999). Activating SRC mutation in a subset of advanced human colon cancers. *Nat Genet* **21**, 187-90.

Irby, R. B. and Yeatman, T. J. (2000). Role of Src expression and activation in human cancer. *Oncogene* **19**, 5636-42.

Jin, L., Wang, W. and Fang, G. (2014). Targeting protein-protein interaction by small molecules. *Annu Rev Pharmacol Toxicol* **54**, 435-56.

Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G. and Winkler, J. (1995). The molecular architecture of focal adhesions. *Annu Rev Cell Dev Biol* **11**, 379-416.

Johnston, I. M., Spence, H. J., Winnie, J. N., McGarry, L., Vass, J. K., Meagher, L., Stapleton, G. and Ozanne, B. W. (2000). Regulation of a multigenic invasion programme by the transcription factor, AP-1: re-expression of a down-regulated gene, TSC-36, inhibits invasion. *Oncogene* **19**, 5348-58.

Kihara, A., Kabeya, Y., Ohsumi, Y. and Yoshimori, T. (2001). Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep* **2**, 330-5.

Koorstra, J. B., Karikari, C. A., Feldmann, G., Bisht, S., Rojas, P. L., Offerhaus, G. J., Alvarez, H. and Maitra, A. (2009). The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. *Cancer Biol Ther* **8**, 618-26.

Korade-Mirnics, Z. and Corey, S. J. (2000). Src kinase-mediated signaling in leukocytes. *J Leukoc Biol* **68**, 603-13.

Korshunov, V. A. (2012). Axl-dependent signalling: a clinical update. *Clin Sci (Lond)* **122**, 361-8.

Krumpe, L. R. and Mori, T. (2007). Potential of phage-displayed peptide library technology to identify functional targeting peptides. *Expert Opin Drug Discov* **2**, 525.

Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., Ohsumi, Y., Tokuhisa, T. and Mizushima, N. (2004). The role of autophagy during the early neonatal starvation period. *Nature* **432**, 1032-6.

Kurenova, E., Liao, J., He, D. H., Hunt, D., Yemma, M., Bshara, W., Seshadri, M. and Cance, W. G. (2013). The FAK scaffold inhibitor C4 disrupts FAK-VEGFR-3 signaling and inhibits pancreatic cancer growth. *Oncotarget* **4**, 1632-46.

LaBaer, J. and Ramachandran, N. (2005). Protein microarrays as tools for functional proteomics. *Curr Opin Chem Biol* **9**, 14-9.

Lark, A. L., Livasy, C. A., Dressler, L., Moore, D. T., Millikan, R. C., Geradts, J., Iacocca, M., Cowan, D., Little, D., Craven, R. J. et al. (2005). High focal adhesion kinase expression in invasive breast carcinomas is associated with an aggressive phenotype. *Mod Pathol* **18**, 1289-94.

Lawson, C., Lim, S. T., Uryu, S., Chen, X. L., Calderwood, D. A. and Schlaepfer, D. D. (2012). FAK promotes recruitment of talin to nascent adhesions to control cell motility. *J Cell Biol* **196**, 223-32.

Le Boeuf, F., Houle, F., Sussman, M. and Huot, J. (2006). Phosphorylation of focal adhesion kinase (FAK) on Ser732 is induced by rho-dependent kinase and is essential for proline-rich tyrosine kinase-2-mediated phosphorylation of FAK on Tyr407 in response to vascular endothelial growth factor. *Mol Biol Cell* **17**, 3508-20.

Lemke, G. (2013). Biology of the TAM receptors. *Cold Spring Harb Perspect Biol* **5**, a009076.

Lemmon, M. A. and Schlessinger, J. (2010). Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117-34.

Leu, T. H. and Maa, M. C. (2002). Tyr-863 phosphorylation enhances focal adhesion kinase autophosphorylation at Tyr-397. *Oncogene* **21**, 6992-7000.

Leung, E. L., Tam, I. Y., Tin, V. P., Chua, D. T., Sihoe, A. D., Cheng, L. C., Ho, J. C., Chung, L. P. and Wong, M. P. (2009). SRC promotes survival and invasion of lung cancers with epidermal growth factor receptor abnormalities and is a potential candidate for molecular-targeted therapy. *Mol Cancer Res* **7**, 923-32.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B. and Schlessinger, J. (1995). Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* **376**, 737-45.

Li, J., Jia, L., Ma, Z. H., Ma, Q. H., Yang, X. H. and Zhao, Y. F. (2012). Axl glycosylation mediates tumor cell proliferation, invasion and lymphatic metastasis in murine hepatocellular carcinoma. *World J Gastroenterol* **18**, 5369-76.

Li, Y., Ye, X., Tan, C., Hongo, J. A., Zha, J., Liu, J., Kallop, D., Ludlam, M. J. and Pei, L. (2009). Axl as a potential therapeutic target in cancer: role of Axl in tumor growth, metastasis and angiogenesis. *Oncogene* **28**, 3442-55.

Liang, C., Feng, P., Ku, B., Dotan, I., Canaani, D., Oh, B. H. and Jung, J. U. (2006). Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol* **8**, 688-99.

Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H. and Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* **402**, 672-6.

Lietha, D., Cai, X., Ceccarelli, D. F., Li, Y., Schaller, M. D. and Eck, M. J. (2007). Structural basis for the autoinhibition of focal adhesion kinase. *Cell* **129**, 1177-87.

Lim, S. T., Chen, X. L., Lim, Y., Hanson, D. A., Vo, T. T., Howerton, K., Larocque, N., Fisher, S. J., Schlaepfer, D. D. and Ilic, D. (2008). Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. *Mol Cell* **29**, 9-22.

Lim, Y., Han, I., Jeon, J., Park, H., Bahk, Y. Y. and Oh, E. S. (2004). Phosphorylation of focal adhesion kinase at tyrosine 861 is crucial for Ras transformation of fibroblasts. *J Biol Chem* **279**, 29060-5.

Linder, S. (2007). The matrix corroded: podosomes and invadopodia in extracellular matrix degradation. *Trends Cell Biol* **17**, 107-17.

Lipinski, C. A. and Loftus, J. C. (2010). Targeting Pyk2 for therapeutic intervention. *Expert Opin Ther Targets* **14**, 95-108.

Liu, E., Cote, J. F. and Vuori, K. (2003). Negative regulation of FAK signaling by SOCS proteins. *EMBO J* **22**, 5036-46.

Liu, T. J., LaFortune, T., Honda, T., Ohmori, O., Hatakeyama, S., Meyer, T., Jackson, D., de Groot, J. and Yung, W. K. (2007). Inhibition of both focal adhesion kinase and insulin-like growth factor-I receptor kinase suppresses glioma proliferation in vitro and in vivo. *Mol Cancer Ther* **6**, 1357-67.

Liu, Y., Loijens, J. C., Martin, K. H., Karginov, A. V. and Parsons, J. T. (2002). The association of ASAP1, an ADP ribosylation factor-GTPase activating protein, with focal adhesion kinase contributes to the process of focal adhesion assembly. *Mol Biol Cell* **13**, 2147-56.

Loges, S., Schmidt, T. and Carmeliet, P. (2010). Mechanisms of resistance to anti-angiogenic therapy and development of third-generation anti-angiogenic drug candidates. *Genes Cancer* **1**, 12-25.

Lu, Y., Zi, X. and Pollak, M. (2004). Molecular mechanisms underlying IGF-I-induced attenuation of the growth-inhibitory activity of trastuzumab (Herceptin) on SKBR3 breast cancer cells. *Int J Cancer* **108**, 334-41.

Lu, Z., Jiang, G., Blume-Jensen, P. and Hunter, T. (2001). Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase. *Mol Cell Biol* **21**, 4016-31.

Ma, A., Richardson, A., Schaefer, E. M. and Parsons, J. T. (2001). Serine phosphorylation of focal adhesion kinase in interphase and mitosis: a possible role in modulating binding to p130(Cas). *Mol Biol Cell* **12**, 1-12.

Maejima, Y., Kyoi, S., Zhai, P., Liu, T., Li, H., Ivessa, A., Sciarretta, S., Del Re, D. P., Zablocki, D. K., Hsu, C. P. et al. (2013). Mst1 inhibits autophagy by promoting the interaction between Beclin1 and Bcl-2. *Nat Med* **19**, 1478-88.

Magnuson, B., Ekim, B. and Fingar, D. C. (2012). Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochem J* **441**, 1-21.

Marino, G., Niso-Santano, M., Baehrecke, E. H. and Kroemer, G. (2014). Self-consumption: the interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol* **15**, 81-94.

Martin, G. S. (1970). Rous sarcoma virus: a function required for the maintenance of the transformed state. *Nature* **227**, 1021-3.

Maruyama, I. N. (2014). Mechanisms of activation of receptor tyrosine kinases: monomers or dimers. *Cells* **3**, 304-30.

Matsunaga, K., Saitoh, T., Tabata, K., Omori, H., Satoh, T., Kurotori, N., Maejima, I., Shirahama-Noda, K., Ichimura, T., Isobe, T. et al. (2009). Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol* **11**, 385-96.

McLean, G. W., Brown, K., Arbuckle, M. I., Wyke, A. W., Pikkarainen, T., Ruoslahti, E. and Frame, M. C. (2001). Decreased focal adhesion kinase suppresses papilloma formation during experimental mouse skin carcinogenesis. *Cancer Res* **61**, 8385-9.

McLean, G. W., Komiyama, N. H., Serrels, B., Asano, H., Reynolds, L., Conti, F., Hodivala-Dilke, K., Metzger, D., Chambon, P., Grant, S. G. et al. (2004). Specific deletion of focal adhesion kinase suppresses tumor formation and blocks malignant progression. *Genes Dev* **18**, 2998-3003.

Meng, X. N., Jin, Y., Yu, Y., Bai, J., Liu, G. Y., Zhu, J., Zhao, Y. Z., Wang, Z., Chen, F., Lee, K. Y. et al. (2009). Characterisation of fibronectin-mediated FAK signalling pathways in lung cancer cell migration and invasion. *Br J Cancer* **101**, 327-34.

Meric, F., Lee, W. P., Sahin, A., Zhang, H., Kung, H. J. and Hung, M. C. (2002). Expression profile of tyrosine kinases in breast cancer. *Clin Cancer Res* **8**, 361-7.

Messina, S., Onofri, F., Bongiorno-Borbone, L., Giovedi, S., Valtorta, F., Girault, J. A. and Benfenati, F. (2003). Specific interactions of neuronal focal adhesion kinase isoforms with Src kinases and amphiphysin. *J Neurochem* **84**, 253-65.

Mitra, S. K., Hanson, D. A. and Schlaepfer, D. D. (2005). Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* **6**, 56-68.

Mitra, S. K., Lim, S. T., Chi, A. and Schlaepfer, D. D. (2006a). Intrinsic focal adhesion kinase activity controls orthotopic breast carcinoma metastasis via the regulation of urokinase plasminogen activator expression in a syngeneic tumor model. *Oncogene* **25**, 4429-40.

Mitra, S. K., Mikolon, D., Molina, J. E., Hsia, D. A., Hanson, D. A., Chi, A., Lim, S. T., Bernard-Trifilo, J. A., Ilic, D., Stupack, D. G. et al. (2006b). Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. *Oncogene* **25**, 5969-84.

Mizutani, K., Ito, H., Iwamoto, I., Morishita, R., Deguchi, T., Nozawa, Y., Asano, T. and Nagata, K. I. (2007). Essential roles of ERK-mediated

phosphorylation of vinexin in cell spreading, migration and anchorage-independent growth. *Oncogene* **26**, 7122-31.

Moja, L., Tagliabue, L., Balduzzi, S., Parmelli, E., Pistotti, V., Guarneri, V. and D'Amico, R. (2012). Trastuzumab containing regimens for early breast cancer. *Cochrane Database Syst Rev* **4**, CD006243.

Molina, M. A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J. and Baselga, J. (2001). Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res* **61**, 4744-9.

Murphy, D. A. and Courtneidge, S. A. (2011). The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. *Nat Rev Mol Cell Biol* **12**, 413-26.

Nagata, Y., Lan, K. H., Zhou, X., Tan, M., Esteva, F. J., Sahin, A. A., Klos, K. S., Li, P., Monia, B. P., Nguyen, N. T. et al. (2004). PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* **6**, 117-27.

Nazio, F., Strappazzon, F., Antonioli, M., Bielli, P., Cianfanelli, V., Bordi, M., Gretzmeier, C., Dengjel, J., Piacentini, M., Fimia, G. M. et al. (2013). mTOR inhibits autophagy by controlling ULK1 ubiquitylation, self-association and function through AMBRA1 and TRAF6. *Nat Cell Biol* **15**, 406-16.

Neet, K. and Hunter, T. (1996). Vertebrate non-receptor protein-tyrosine kinase families. *Genes Cells* **1**, 147-69.

Nero, T. L., Morton, C. J., Holien, J. K., Wielens, J. and Parker, M. W. (2014). Oncogenic protein interfaces: small molecules, big challenges. *Nat Rev Cancer* **14**, 248-62.

Nitta, T., Sato, Y., Ren, X. S., Harada, K., Sasaki, M., Hirano, S. and Nakanuma, Y. (2014). Autophagy may promote carcinoma cell invasion and correlate with poor prognosis in cholangiocarcinoma. *Int J Clin Exp Pathol* **7**, 4913-21.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Nolan, K., Lacoste, J. and Parsons, J. T. (1999). Regulated expression of focal adhesion kinase-related nonkinase, the autonomously expressed C-terminal domain of focal adhesion kinase. *Mol Cell Biol* **19**, 6120-9.

Nooren, I. M. and Thornton, J. M. (2003). Diversity of protein-protein interactions. *EMBO J* **22**, 3486-92.

Okada, M. (2012). Regulation of the SRC family kinases by Csk. *Int J Biol Sci* **8**, 1385-97.

Olayioye, M. A., Graus-Porta, D., Beerli, R. R., Rohrer, J., Gay, B. and Hynes, N. E. (1998). ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. *Mol Cell Biol* **18**, 5042-51.

Otey, C. A. (1998). A role for pp125FAK in suppression of apoptosis in fibroblasts. *Biol Bull* **194**, 387-8; discussion 388-9.

Owen, J. D., Ruest, P. J., Fry, D. W. and Hanks, S. K. (1999). Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. *Mol Cell Biol* **19**, 4806-18.

Ozbabacan, S. E., Engin, H. B., Gursoy, A. and Keskin, O. (2011). Transient protein-protein interactions. *Protein Eng Des Sel* **24**, 635-48.

Paccez, J. D., Vogelsang, M., Parker, M. I. and Zerbini, L. F. (2014). The receptor tyrosine kinase Axl in cancer: biological functions and therapeutic implications. *Int J Cancer* **134**, 1024-33.

Panaretou, C., Domin, J., Cockcroft, S. and Waterfield, M. D. (1997). Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150.Ptdins 3-kinase complex. *J Biol Chem* **272**, 2477-85.

Pande, J., Szewczyk, M. M. and Grover, A. K. (2010). Phage display: concept, innovations, applications and future. *Biotechnol Adv* **28**, 849-58.

Pandolfi, P. P. (2004). Breast cancer--loss of PTEN predicts resistance to treatment. *N Engl J Med* **351**, 2337-8.

Pankova, K., Rosel, D., Novotny, M. and Brabek, J. (2010). The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells. *Cell Mol Life Sci* **67**, 63-71.

Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M. and Weed, S. A. (2000). Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* **19**, 5606-13.

Parsons, S. J. and Parsons, J. T. (2004). Src family kinases, key regulators of signal transduction. *Oncogene* **23**, 7906-9.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D. and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122**, 927-39.

Peng, X., Ueda, H., Zhou, H., Stokol, T., Shen, T. L., Alcaraz, A., Nagy, T., Vassalli, J. D. and Guan, J. L. (2004). Overexpression of focal adhesion kinase in vascular endothelial cells promotes angiogenesis in transgenic mice. *Cardiovasc Res* **64**, 421-30.

Perkins, J. R., Diboun, I., Dessailly, B. H., Lees, J. G. and Orengo, C. (2010). Transient protein-protein interactions: structural, functional, and network properties. *Structure* **18**, 1233-43.

Petricevic, B., Laengle, J., Singer, J., Sachet, M., Fazekas, J., Steger, G., Bartsch, R., Jensen-Jarolim, E. and Bergmann, M. (2013). Trastuzumab mediates antibody-dependent cell-mediated cytotoxicity and phagocytosis to the same extent

in both adjuvant and metastatic HER2/neu breast cancer patients. *J Transl Med* **11**, 307.

Plaza-Menacho, I., Morandi, A., Mologni, L., Boender, P., Gambacorti-Passerini, C., Magee, A. I., Hofstra, R. M., Knowles, P., McDonald, N. Q. and Isacke, C. M. (2011). Focal Adhesion Kinase (FAK) Binds RET Kinase via Its FERM Domain, Priming a Direct and Reciprocal RET-FAK Transactivation Mechanism. *J Biol Chem* **286**, 17292-302.

Pohlmann, P. R., Mayer, I. A. and Mernaugh, R. (2009). Resistance to Trastuzumab in Breast Cancer. *Clin Cancer Res* **15**, 7479-7491.

Polte, T. R. and Hanks, S. K. (1995). Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. *Proc Natl Acad Sci U S A* **92**, 10678-82.

Polte, T. R., Naftilan, A. J. and Hanks, S. K. (1994). Focal adhesion kinase is abundant in developing blood vessels and elevation of its phosphotyrosine content in vascular smooth muscle cells is a rapid response to angiotensin II. *J Cell Biochem* **55**, 106-19.

Portt, L., Norman, G., Clapp, C., Greenwood, M. and Greenwood, M. T. (2011). Anti-apoptosis and cell survival: a review. *Biochim Biophys Acta* **1813**, 238-59.

Price-Schiavi, S. A., Jepson, S., Li, P., Arango, M., Rudland, P. S., Yee, L. and Carraway, K. L. (2002). Rat Muc4 (sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cell surfaces, a potential mechanism for herceptin resistance. *Int J Cancer* **99**, 783-91.

Pylayeva, Y., Gillen, K. M., Gerald, W., Beggs, H. E., Reichardt, L. F. and Giancotti, F. G. (2009). Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling. *J Clin Invest* **119**, 252-66.

Quintas-Cardama, A., Qiu, Y. H., Post, S. M., Zhang, Y., Creighton, C. J., Cortes, J. and Kornblau, S. M. (2012). Reverse phase protein array profiling reveals distinct proteomic signatures associated with chronic myeloid leukemia progression and with chronic phase in the CD34-positive compartment. *Cancer* **118**, 5283-92.

Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. F. and Chen, H. C. (1999). Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. *J Biol Chem* **274**, 12361-6.

Ren, X. D., Kiosses, W. B., Sieg, D. J., Otey, C. A., Schlaepfer, D. D. and Schwartz, M. A. (2000). Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J Cell Sci* **113 (Pt 20)**, 3673-8.

Ribon, V., Herrera, R., Kay, B. K. and Saltiel, A. R. (1998). A role for CAP, a novel, multifunctional Src homology 3 domain-containing protein in formation of actin stress fibers and focal adhesions. *J Biol Chem* **273**, 4073-80.

Richardson, A. and Parsons, T. (1996). A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK. *Nature* **380**, 538-40.

Roberts, W. G., Ung, E., Whalen, P., Cooper, B., Hulford, C., Autry, C., Richter, D., Emerson, E., Lin, J., Kath, J. et al. (2008). Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. *Cancer Res* **68**, 1935-44.

Robinson, D. R., Wu, Y. M. and Lin, S. F. (2000). The protein tyrosine kinase family of the human genome. *Oncogene* **19**, 5548-57.

Romer, L. H., McLean, N., Turner, C. E. and Burridge, K. (1994). Tyrosine kinase activity, cytoskeletal organization, and motility in human vascular endothelial cells. *Mol Biol Cell* **5**, 349-61.

Rosen, L. (2000). Antiangiogenic strategies and agents in clinical trials. *Oncologist* **5 Suppl 1**, 20-7.

Roussel, R. R., Brodeur, S. R., Shalloway, D. and Laudano, A. P. (1991). Selective binding of activated pp60c-src by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60c-src. *Proc Natl Acad Sci U S A* **88**, 10696-700.

Sahai, E. and Marshall, C. J. (2003). Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol* **5**, 711-9.

Sainaghi, P. P., Castello, L., Bergamasco, L., Galletti, M., Bellosta, P. and Avanzi, G. C. (2005). Gas6 induces proliferation in prostate carcinoma cell lines expressing the Axl receptor. *J Cell Physiol* **204**, 36-44.

Sakurama, K., Noma, K., Takaoka, M., Tomono, Y., Watanabe, N., Hatakeyama, S., Ohmori, O., Hirota, S., Motoki, T., Shirakawa, Y. et al. (2009). Inhibition of focal adhesion kinase as a potential therapeutic strategy for imatinib-resistant gastrointestinal stromal tumor. *Mol Cancer Ther* **8**, 127-34.

Sandilands, E., Serrels, B., McEwan, D. G., Morton, J. P., Macagno, J. P., McLeod, K., Stevens, C., Brunton, V. G., Langdon, W. Y., Vidal, M. et al. (2012a). Autophagic targeting of Src promotes cancer cell survival following reduced FAK signalling. *Nat Cell Biol* **14**, 51-60.

Sandilands, E., Serrels, B., Wilkinson, S. and Frame, M. C. (2012b). Src-dependent autophagic degradation of Ret in FAK-signalling-defective cancer cells. *EMBO Rep* **13**, 733-40.

Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K. and Sasaki, T. (1995). Cloning and characterization of cell adhesion kinase beta, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *J Biol Chem* **270**, 21206-19.

Sato, H., Takino, T. and Miyamori, H. (2005). Roles of membrane-type matrix metalloproteinase-1 in tumor invasion and metastasis. *Cancer Sci* **96**, 212-7.

Sawabu, T., Seno, H., Kawashima, T., Fukuda, A., Uenoyama, Y., Kawada, M., Kanda, N., Sekikawa, A., Fukui, H., Yanagita, M. et al. (2007). Growth arrest-specific gene 6 and Axl signaling enhances gastric cancer cell survival via Akt pathway. *Mol Carcinog* **46**, 155-64.

Sayan, A. E., Stanford, R., Vickery, R., Grigorenko, E., Diesch, J., Kulbicki, K., Edwards, R., Pal, R., Greaves, P., Jariel-Encontre, I. et al. (2012). Fra-1 controls motility of bladder cancer cells via transcriptional upregulation of the receptor tyrosine kinase AXL. *Oncogene* **31**, 1493-503.

Schaller, M. D. (2010). Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *J Cell Sci* **123**, 1007-13.

Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. (1992). pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci U S A* **89**, 5192-6.

Schaller, M. D., Borgman, C. A. and Parsons, J. T. (1993). Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK. *Mol Cell Biol* **13**, 785-91.

Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R. and Parsons, J. T. (1994). Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* **14**, 1680-8.

Schlaepfer, D. D., Hanks, S. K., Hunter, T. and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372**, 786-91.

Schlaepfer, D. D., Hauck, C. R. and Sieg, D. J. (1999). Signaling through focal adhesion kinase. *Prog Biophys Mol Biol* **71**, 435-78.

Schlaepfer, D. D. and Hunter, T. (1996). Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol* **16**, 5623-33.

Schlaepfer, D. D. and Mitra, S. K. (2004). Multiple connections link FAK to cell motility and invasion. *Curr Opin Genet Dev* **14**, 92-101.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. *Cell* **103**, 211-25.

Schlessinger, J. and Lemmon, M. A. (2003). SH2 and PTB domains in tyrosine kinase signaling. *Sci STKE* **2003**, RE12.

Schoenlein, P. V., Periyasamy-Thandavan, S., Samaddar, J. S., Jackson, W. H. and Barrett, J. T. (2009). Autophagy facilitates the progression of ERalpha-positive breast cancer cells to antiestrogen resistance. *Autophagy* **5**, 400-3.

Sen, B. and Johnson, F. M. (2011). Regulation of SRC family kinases in human cancers. *J Signal Transduct* **2011**, 865819.

Serrels, A., McLeod, K., Canel, M., Kinnaird, A., Graham, K., Frame, M. C. and Brunton, V. G. (2012). The role of focal adhesion kinase catalytic activity on the proliferation and migration of squamous cell carcinoma cells. *Int J Cancer* **131**, 287-97.

Serrels, B., Sandilands, E., Serrels, A., Baillie, G., Houslay, M. D., Brunton, V. G., Canel, M., Machesky, L. M., Anderson, K. I. and Frame, M. C. (2010). A complex between FAK, RACK1, and PDE4D5 controls spreading initiation and cancer cell polarity. *Curr Biol* **20**, 1086-92.

Serrels, B., Serrels, A., Brunton, V. G., Holt, M., McLean, G. W., Gray, C. H., Jones, G. E. and Frame, M. C. (2007). Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. *Nat Cell Biol* **9**, 1046-56.

Sgambato, A., Cittadini, A., Faraglia, B. and Weinstein, I. B. (2000). Multiple functions of p27(Kip1) and its alterations in tumor cells: a review. *J Cell Physiol* **183**, 18-27.

Shah, N. P., Nicoll, J. M., Nagar, B., Gorre, M. E., Paquette, R. L., Kuriyan, J. and Sawyers, C. L. (2002). Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* **2**, 117-25.

Shattuck, D. L., Miller, J. K., Carraway, K. L., 3rd and Sweeney, C. (2008). Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. *Cancer Res* **68**, 1471-7.

Shawver, L. K., Slamon, D. and Ullrich, A. (2002). Smart drugs: tyrosine kinase inhibitors in cancer therapy. *Cancer Cell* **1**, 117-23.

Shen, T. L., Park, A. Y., Alcaraz, A., Peng, X., Jang, I., Koni, P., Flavell, R. A., Gu, H. and Guan, J. L. (2005). Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. *J Cell Biol* **169**, 941-52.

Shi, Q., Hjelmeland, A. B., Keir, S. T., Song, L., Wickman, S., Jackson, D., Ohmori, O., Bigner, D. D., Friedman, H. S. and Rich, J. N. (2007). A novel low-molecular weight inhibitor of focal adhesion kinase, TAE226, inhibits glioma growth. *Mol Carcinog* **46**, 488-96.

Shieh, Y. S., Lai, C. Y., Kao, Y. R., Shiah, S. G., Chu, Y. W., Lee, H. S. and Wu, C. W. (2005). Expression of axl in lung adenocarcinoma and correlation with tumor progression. *Neoplasia* **7**, 1058-64.

Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H. and Schlaepfer, D. D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol* **2**, 249-56.

Sieg, D. J., Ilic, D., Jones, K. C., Damsky, C. H., Hunter, T. and Schlaepfer, D. D. (1998). Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK- cell migration. *EMBO J* **17**, 5933-47.

Smith, M. R., DeGudicibus, S. J. and Stacey, D. W. (1986). Requirement for c-ras proteins during viral oncogene transformation. *Nature* **320**, 540-3.

Sonoda, Y., Matsumoto, Y., Funakoshi, M., Yamamoto, D., Hanks, S. K. and Kasahara, T. (2000). Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem* **275**, 16309-15.

Sordella, R., Bell, D. W., Haber, D. A. and Settleman, J. (2004). Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* **305**, 1163-7.

Stehelin, D., Varmus, H. E., Bishop, J. M. and Vogt, P. K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* **260**, 170-3.

Stokes, J. B., Adair, S. J., Slack-Davis, J. K., Walters, D. M., Tilghman, R. W., Hershey, E. D., Lowrey, B., Thomas, K. S., Bouton, A. H., Hwang, R. F. et al. (2011). Inhibition of focal adhesion kinase by PF-562,271 inhibits the growth and metastasis of pancreatic cancer concomitant with altering the tumor microenvironment. *Mol Cancer Ther* **10**, 2135-45.

Stynen, B., Tournu, H., Tavernier, J. and Van Dijck, P. (2012). Diversity in genetic in vivo methods for protein-protein interaction studies: from the yeast two-hybrid system to the mammalian split-luciferase system. *Microbiol Mol Biol Rev* **76**, 331-82.

Sugimoto, Y., Whitman, M., Cantley, L. C. and Erikson, R. L. (1984). Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. *Proc Natl Acad Sci U S A* **81**, 2117-21.

Tachibana, K., Sato, T., D'Avirro, N. and Morimoto, C. (1995). Direct association of pp125FAK with paxillin, the focal adhesion-targeting mechanism of pp125FAK. *J Exp Med* **182**, 1089-99.

Tai, K. Y., Shieh, Y. S., Lee, C. S., Shiah, S. G. and Wu, C. W. (2008). Axl promotes cell invasion by inducing MMP-9 activity through activation of NF-kappaB and Brg-1. *Oncogene* **27**, 4044-55.

Tait, S. W. and Green, D. R. (2008). Caspase-independent cell death: leaving the set without the final cut. *Oncogene* **27**, 6452-61.

Takahashi, Y., Coppola, D., Matsushita, N., Cualing, H. D., Sun, M., Sato, Y., Liang, C., Jung, J. U., Cheng, J. Q., Mule, J. J. et al. (2007). Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* **9**, 1142-51.

Tavora, B., Batista, S., Reynolds, L. E., Jadeja, S., Robinson, S., Kostourou, V., Hart, I., Fruttiger, M., Parsons, M. and Hodivala-Dilke, K. M. (2010). Endothelial FAK is required for tumour angiogenesis. *EMBO Mol Med* **2**, 516-28.

Taylor, S. J. and Shalloway, D. (1996). Src and the control of cell division. *Bioessays* **18**, 9-11.

Theocharis, S. E., Kouraklis, G. P., Kakisis, J. D., Kanelli, H. G., Apostolakou, F. E., Karatzas, G. M. and Koutselinis, A. S. (2003). Focal adhesion kinase expression is not a prognostic predictor in colon adenocarcinoma patients. *Eur J Surg Oncol* **29**, 571-4.

Thomas, S. M. and Brugge, J. S. (1997). Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* **13**, 513-609.

Tilghman, R. W., Slack-Davis, J. K., Sergina, N., Martin, K. H., Iwanicki, M., Hershey, E. D., Beggs, H. E., Reichardt, L. F. and Parsons, J. T. (2005). Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells. *J Cell Sci* **118**, 2613-23.

Tomar, A. and Schlaepfer, D. D. (2009). Focal adhesion kinase: switching between GAPs and GEFs in the regulation of cell motility. *Curr Opin Cell Biol* **21**, 676-83.

Turkson, J., Bowman, T., Garcia, R., Caldenhoven, E., De Groot, R. P. and Jove, R. (1998). Stat3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol Cell Biol* **18**, 2545-52.

Ucar, D. A., Kurenova, E., Garrett, T. J., Cance, W. G., Nyberg, C., Cox, A., Massoll, N., Ostrov, D. A., Lawrence, N., Sebti, S. M. et al. (2012). Disruption of the protein interaction between FAK and IGF-1R inhibits melanoma tumor growth. *Cell Cycle* **11**, 3250-9.

Ullrich, A. and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-12.

van Miltenburg, M. H., van Nimwegen, M. J., Tijdens, I., Lalai, R., Kuiper, R., Klarenbeek, S., Schouten, P. C., de Vries, A., Jonkers, J. and van de Water, B. (2014). Mammary gland-specific ablation of focal adhesion kinase reduces the incidence of p53-mediated mammary tumour formation. *Br J Cancer* **110**, 2747-55.

Vasioukhin, V., Degenstein, L., Wise, B. and Fuchs, E. (1999). The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. *Proc Natl Acad Sci U S A* **96**, 8551-6.

Verma, A., Warner, S. L., Vankayalapati, H., Bearss, D. J. and Sharma, S. (2011). Targeting Axl and Mer kinases in cancer. *Mol Cancer Ther* **10**, 1763-73.

Vodnik, M., Zager, U., Strukelj, B. and Lunder, M. (2011). Phage display: selecting straws instead of a needle from a haystack. *Molecules* **16**, 790-817.

Vuoriluoto, K., Haugen, H., Kiviluoto, S., Mpindi, J. P., Nevo, J., Gjerdrum, C., Tiron, C., Lorens, J. B. and Ivaska, J. (2011). Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. *Oncogene* **30**, 1436-48.

Walker, S., Chandra, P., Manifava, M., Axe, E. and Ktistakis, N. T. (2008). Making autophagosomes: localized synthesis of phosphatidylinositol 3-phosphate holds the clue. *Autophagy* **4**, 1093-6.

Wang, D., Grammer, J. R., Cobbs, C. S., Stewart, J. E., Jr., Liu, Z., Rhoden, R., Hecker, T. P., Ding, Q. and Gladson, C. L. (2000). p125 focal adhesion kinase promotes malignant astrocytoma cell proliferation in vivo. *J Cell Sci* **113 Pt 23**, 4221-30.

Wang, R. C., Wei, Y., An, Z., Zou, Z., Xiao, G., Bhagat, G., White, M., Reichelt, J. and Levine, B. (2012). Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. *Science* **338**, 956-9.

Wang, Y. and McNiven, M. A. (2012). Invasive matrix degradation at focal adhesions occurs via protease recruitment by a FAK-p130Cas complex. *J Cell Biol* **196**, 375-85.

Wang, Z. G., Fukazawa, T., Nishikawa, T., Watanabe, N., Sakurama, K., Motoki, T., Takaoka, M., Hatakeyama, S., Omori, O., Ohara, T. et al. (2008). TAE226, a dual inhibitor for FAK and IGF-IR, has inhibitory effects on mTOR signaling in esophageal cancer cells. *Oncol Rep* **20**, 1473-7.

Watermann, D. O., Gabriel, B., Jager, M., Orlowska-Volk, M., Hasenburg, A., zur Hausen, A., Gitsch, G. and Stickeler, E. (2005). Specific induction of pp125 focal adhesion kinase in human breast cancer. *Br J Cancer* **93**, 694-8.

Weis, S. M., Lim, S. T., Lutu-Fuga, K. M., Barnes, L. A., Chen, X. L., Gothert, J. R., Shen, T. L., Guan, J. L., Schlaepfer, D. D. and Cheresh, D. A. (2008). Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK. *J Cell Biol* **181**, 43-50.

Wendt, M. K. and Schiemann, W. P. (2009). Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-beta signaling and metastasis. *Breast Cancer Res* **11**, R68.

Westhoff, M. A., Serrels, B., Fincham, V. J., Frame, M. C. and Carragher, N. O. (2004). SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol Cell Biol* **24**, 8113-33.

Whitney, G. S., Chan, P. Y., Blake, J., Cosand, W. L., Neubauer, M. G., Aruffo, A. and Kanner, S. B. (1993). Human T and B lymphocytes express a structurally conserved focal adhesion kinase, pp125FAK. *DNA Cell Biol* **12**, 823-30.

Willats, W. G. (2002). Phage display: practicalities and prospects. *Plant Mol Biol* **50**, 837-54.

Wu, C. W., Li, A. F., Chi, C. W., Lai, C. H., Huang, C. L., Lo, S. S., Lui, W. Y. and Lin, W. C. (2002). Clinical significance of AXL kinase family in gastric cancer. *Anticancer Res* **22**, 1071-8.

Wu, J., Liao, X., Yu, B. and Su, B. (2013). Dasatinib inhibits primary melanoma cell proliferation through morphology-dependent disruption of Src-ERK signaling. *Oncol Lett* **5**, 527-532.

Wu, X., Gan, B., Yoo, Y. and Guan, J. L. (2005). FAK-mediated src phosphorylation of endophilin A2 inhibits endocytosis of MT1-MMP and promotes ECM degradation. *Dev Cell* **9**, 185-96.

Wu, X., Suetsugu, S., Cooper, L. A., Takenawa, T. and Guan, J. L. (2004). Focal adhesion kinase regulation of N-WASP subcellular localization and function. *J Biol Chem* **279**, 9565-76.

Wu, Z. M., Yuan, X. H., Jiang, P. C., Li, Z. Q. and Wu, T. (2006). Antisense oligonucleodes targeting the focal adhesion kinase inhibit proliferation, induce apoptosis and cooperate with cytotoxic drugs in human glioma cells. *J Neurooncol* **77**, 117-23.

Xing, Z., Chen, H. C., Nowlen, J. K., Taylor, S. J., Shalloway, D. and Guan, J. L. (1994). Direct interaction of v-Src with the focal adhesion kinase mediated by the Src SH2 domain. *Mol Biol Cell* **5**, 413-21.

Xiong, W. C., Macklem, M. and Parsons, J. T. (1998). Expression and characterization of splice variants of PYK2, a focal adhesion kinase-related protein. *J Cell Sci* **111 (Pt 14)**, 1981-91.

Xu, L., Liu, T., Han, F., Zong, Z., Wang, G., Yu, B. and Zhang, J. (2012). AURKB and MAPK involvement in the regulation of the early stages of mouse zygote development. *Sci China Life Sci* **55**, 47-56.

Xu, W., Harrison, S. C. and Eck, M. J. (1997). Three-dimensional structure of the tyrosine kinase c-Src. *Nature* **385**, 595-602.

Yang, Z. and Klionsky, D. J. (2010). Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* **22**, 124-31.

Ye, F., Hu, G., Taylor, D., Ratnikov, B., Bobkov, A. A., McLean, M. A., Sligar, S. G., Taylor, K. A. and Ginsberg, M. H. (2010a). Recreation of the terminal events in physiological integrin activation. *J Cell Biol* **188**, 157-73.

Ye, X., Li, Y., Stawicki, S., Couto, S., Eastham-Anderson, J., Kallop, D., Weimer, R., Wu, Y. and Pei, L. (2010b). An anti-Axl monoclonal antibody attenuates xenograft tumor growth and enhances the effect of multiple anticancer therapies. *Oncogene* **29**, 5254-64.

Yu, H., Li, X., Marchetto, G. S., Dy, R., Hunter, D., Calvo, B., Dawson, T. L., Wilm, M., Anderegg, R. J., Graves, L. M. et al. (1996). Activation of a novel calcium-dependent protein-tyrosine kinase. Correlation with c-Jun N-terminal kinase but not mitogen-activated protein kinase activation. *J Biol Chem* **271**, 29993-8.

Zeng, L., Si, X., Yu, W. P., Le, H. T., Ng, K. P., Teng, R. M., Ryan, K., Wang, D. Z., Ponniah, S. and Pallen, C. J. (2003). PTP alpha regulates integrin-stimulated FAK autophosphorylation and cytoskeletal rearrangement in cell spreading and migration. *J Cell Biol* **160**, 137-46.

Zhang, J., Yang, P. L. and Gray, N. S. (2009). Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* **9**, 28-39.

Zhang, L., Teng, Y., Zhang, Y., Liu, J., Xu, L., Qu, J., Hou, K., Yang, X., Liu, Y. and Qu, X. (2012). c-Src expression is predictive of poor prognosis in breast cancer patients with bone metastasis, but not in patients with visceral metastasis. *APMIS* **120**, 549-57.

Zhang, X., Chattopadhyay, A., Ji, Q. S., Owen, J. D., Ruest, P. J., Carpenter, G. and Hanks, S. K. (1999). Focal adhesion kinase promotes phospholipase C-gamma1 activity. *Proc Natl Acad Sci U S A* **96**, 9021-6.

Zhang, Y., Tang, Y. J., Man, Y., Pan, F., Li, Z. H. and Jia, L. S. (2013). Knockdown of AXL receptor tyrosine kinase in osteosarcoma cells leads to decreased proliferation and increased apoptosis. *Int J Immunopathol Pharmacol* **26**, 179-88.

Zhang, Y. X., Knyazev, P. G., Cheburkin, Y. V., Sharma, K., Knyazev, Y. P., Orfi, L., Szabadkai, I., Daub, H., Keri, G. and Ullrich, A. (2008). AXL is a potential target for therapeutic intervention in breast cancer progression. *Cancer Res* **68**, 1905-15.

Zhao, J., Bian, Z. C., Yee, K., Chen, B. P., Chien, S. and Guan, J. L. (2003). Identification of transcription factor KLF8 as a downstream target of focal adhesion kinase in its regulation of cyclin D1 and cell cycle progression. *Mol Cell* **11**, 1503-15.

Zhao, J., Pestell, R. and Guan, J. L. (2001). Transcriptional activation of cyclin D1 promoter by FAK contributes to cell cycle progression. *Mol Biol Cell* **12**, 4066-77.

Zheng, X. M., Wang, Y. and Pallen, C. J. (1992). Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase. *Nature* **359**, 336-9.

Zheng, Y., Xia, Y., Hawke, D., Halle, M., Tremblay, M. L., Gao, X., Zhou, X. Z., Aldape, K., Cobb, M. H., Xie, K. et al. (2009). FAK phosphorylation by ERK primes ras-induced tyrosine dephosphorylation of FAK mediated by PIN1 and PTP-PEST. *Mol Cell* **35**, 11-25.

Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T. et al. (2001). Global analysis of protein activities using proteome chips. *Science* **293**, 2101-5.

Zhu, H. and Snyder, M. (2003). Protein chip technology. *Curr Opin Chem Biol* **7**, 55-63.

Zinzalla, G. and Thurston, D. E. (2009). Targeting protein-protein interactions for therapeutic intervention: a challenge for the future. *Future Med Chem* **1**, 65-93.

Zwick, E., Bange, J. and Ullrich, A. (2002). Receptor tyrosine kinases as targets for anticancer drugs. *Trends Mol Med* **8**, 17-23.